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Following exposure to the $P2X_7$ receptor agonist benzyl ATP (BzATP) or to HD, CHO-K1 cells underwent cell death which was largely apoptotic in nature. The toxicity was very dependant on the ionic composition of the incubation medium. Inhibition of the $P2X_7$ receptor by oxidized ATP (oATP) blocked BzATP induced cytotoxicity but appeared only to shunt HD induced cell death from apoptosis (as measured by a variety of techniques) to necrosis.

A membrane bound, cytolytic pore complex was identified in CHO-K1 cells which consisted of two separate receptors coupled to a cytolytic pore. Agonists of the two receptors, factors controlling the activation of the cytolytic pore and the fate of the cell following pore activation were examined. Initial studies concerning the interactions of HD with various constituents of the complex were carried out. HD under the conditions employed to date did not activate the pore directly nor did it promote activation of the $P2X_7$ receptor but studies are ongoing. An extensive study of the role of calcium in HD induced cell death was continued indicating that calcium appears to be necessary for apoptosis but not cell death. A very important role for ions in HD induced cell death was identified

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TABLE OF CONTENTS Page #

## INTRODUCTION

Sulphur mustard (HD) is a toxic chemical warfare (CW) agent that causes lesions on the skin and in a variety of tissues following systemic exposure. The lesions have been referred to as necrotic, but in fact, little is known concerning the mechanism of cell death. The body displays the ability to rid itself of cells it no longer needs to make way for replacement cells or to eliminate damaged ones. This process is referred to as apoptosis (cell suicide) and it can be activated by external stimulae, as well as under normal physiological conditions. Apoptosis and necrosis can be triggered *in vivo* by endogenous mechanisms that employ naturally occurring cytolytic compounds such as ATP. ATP causes cell death in a variety of tissues through activation of cellular membrane receptors known as the P2X purinoceptors. These act as ionotropic receptors that are coupled to a pore, which when activated, allows the entrance of large molecular weight molecules. We have been investigating the possibility that HD might potentiate the endogenous effects of ATP agonists on naturally occurring purinceptors to cause cellular lesions through direct activation of their associated cytolytic pores in the membrane. During the conduct of these studies we have identified a number of interesting mechanisms involved in the activation and control of cell death.

## BACKGROUND

### $P2X_7$ Receptors and HD Induced Cell Death: Role of Pore Dilation

This past year's studies were carried out to determine whether P2X receptor subtypes were capable of playing a role in cell death resulting from HD exposure. CHO-K1 cells were chosen for study since results from our laboratory and those in the literature (Michel *et al.*, 1998) have shown that native CHO-K1 cells contain $P2X_7$ receptors, with little interference from the effects of other P2 receptor sub-types (for a description of the structure and function of the $P2X_7$ receptor see next section). HD was shown to cause a concentration-related increase in cell death, which was documented in a wide variety of cell types, utilizing a variety of tests routinely used to define apoptosis (in last year's report, Gong *et al.*, 2001), including assays of DNA fragmentation and morphology. In order to determine whether the $P2X_7$ receptor played a role in HD induced cell death, it was necessary to demonstrate that stimulation of the receptor by

itself was sufficient to produce cytotoxic effects. However, it was clearly highlighted in last year's report (Fig. 23, pg 18 under problems encountered) that we were not able to show ATP-induced cell death under our experimental conditions. Failure to demonstrate cell death from activation of $P2X_7$ receptors would have resulted in severe problems in testing our theory concerning the effects of HD on P2X receptor types (Lundy *et al.*, 1998, and the subject of this co-operative agreement). Furthermore, several other investigators have reported a definite relationship between activation of these receptors and both apoptotic and necrotic cell death (Pizzo *et al.*, 1991; Dubyak and El-Moatassim, 1993; Murgia *et al.*, 1993; Rassendren *et al.*, 1997). At the time of the submission of last year's report we were not able to repeat these findings.

In the first part of this report we outline the results of experiments carried out to determine what conditions are necessary and what factors interfere with the demonstration of $P2X_7$ stimulation induced cytotoxicity in CHO-K1 cells, as well as other cells by inference. The results in this present report show that by alteration of certain factors, most specifically the ionic composition of the incubation medium, that these problems can be been largely overcome. In the process of examining those factors that affected BzATP activation of the pore linked to the $P2X_7$ receptor, important mechanisms in the control of cytotoxic responses have been documented. One novel finding is the discovery of the co-existence of an additional receptor which, although totally distinct from the $P2X_7$ receptor, is nevertheless directly associated with the same $P2X_7$ linked pore in CHO-K1 cells. Stimulation of this second receptor by its prototypical agonist maitotoxin, appears to result in necrosis, as opposed to $P2X_7$ receptor stimulation, which results primarily in apoptosis. Since we are interested in the interactions of HD on the BzATP operated pore, the discovery of another controlling influence on that pore prompted us to examine it as well. We have therefore included studies on the effects of HD on the $P2X_7$ receptor and its pore, as well as those carried out on the maitotoxin receptor activated pore.

The results in the following sections describe studies that demonstrate $P2X_7$ receptor mediated cell death. They also outline those factors that control the opening of the cytolytic pore from each "side", or from each receptor, in what will be termed the "complex". During these studies, the importance of ionic environment has been

emphasized, both in the cytotoxicity of ATP, and of HD. The role of the divalent cation calcium in HD induced cell death is also investigated. Finally, the results from some additional experiments concerning the presence of $P2X_7$ receptors on nervous structures are included.

**Studies on the BzATP/Maitotoxin Cytolytic/Oncotic Pore: Effect of HD**

The $P2X_7$ receptor is an ionotropic pore which when stimulated, allows the influx of calcium and other cations. The consequence of the initial stimulation of the receptor is the opening of a non-specific membrane pore to which the receptor is coupled. Receptor activation by ATP and certain of its analogues (the most potent being benzyl ATP (BzATP)), results in pore dilation, which allows the entrance into the cytoplasm of molecules of up to about 900 daltons from the extracellular compartment (Wiley *et al.*, 1996). The literature now clearly suggests that activation of this receptor and the subsequent entrance of large molecular weight species initiates cell death (Rassendren *et al.*,1997). It is becoming increasingly clear that activation of the $P2X_7$ receptor followed by the opening of the non-specific pore, initiates caspase induction, PARP cleavage, DNA fragmentation, release of cytokines, activation of phospholipases resulting in apoptosis and under certain conditions also necrosis (Di Virgilio, 1995; Zheng *et al.*, 1991; Ferrari *et al.*, 1999; Coutinho-Silva *et al.*, 1999; Schulze-Lohoff *et al.*, 1998; Humphreys and Dubyak, 1996; Humphreys *et al.*, 2000; Solini *et al.*, 1999). These effects are very much reminiscent of those caused by HD.

Recent studies suggest that in addition to the coupling of the $P2X_7$ receptor to the pore, other mechanisms may stimulate pore opening as well, although no concrete examples have been yet identified. It is therefore an open question as to whether a variety of cytolytic compounds may in fact cause cell death by utilizing this pore as a common site of action. Figure 7 represents an outline of several possible sites of action of cytotoxic compounds using the concept of a cytolytic pore complex. This diagram has been generated in part as the result of work in this laboratory carried out under the terms of the co-operative agreement and which has solidified our understanding of how maitotoxin and $P2X_7$ agonists effect pore opening. The cytolytic pore could be activated through the $P2X_7$ receptor (BzATP). It could also be activated, at least theoretically, by

compounds directly acting on the pore (although no examples have been described yet) or the pore could be activated through coupling with another receptor, such as occurs with maitotoxin. Maitotoxin is one of the most toxic substances ever described and in addition to its extreme acute systemic toxicity, it is a potent cytolytic agent whose toxicity has been reported to be dependent on the influx of external calcium (Kutty *et al.*, 1989). From our point of view these different mechanisms of cytotoxicity suggest some interesting avenues through which HD might cause cell death since the consequences of the opening of the pore and the effects of HD have so very many common effects (see above). On the diagram we have identified within this framework of cytotoxic mechanisms, possible sites that we have or are currently examining, at which we believe HD could act in this multifaceted system to cause cytotoxicity.

In this report we describe experiments that were done to define the relationship between the action of different compounds on some aspect of the $P2X_7$ receptor/maitotoxin receptor/pore complex which leads in one way or another to subsequent cytotoxicity. Experiments are described which define the various factors that control the opening of the pore in response to various stimuli. Descriptions are given of experiments that were conducted in order to determine the effects of HD on the sites within the complex. Since our studies have been carried out to examine the effects of HD on the $P2X_7$ receptor (or the cytolytic pore connected to it), we examined the possibility that HD's target might in fact be this pore, or the $P2X_7$ receptor that controls pore dilation. Since maitotoxin also activates this pore, but by a totally different mechanism, it is conceivable that HD may act on the maitotoxin "side" of the pore, at least under certain conditions to cause it to open and initiate cytotoxicity.

## METHODS

### Cell Culture and Cytotoxicity Assays

**CHO-K1 Cell Line Culture** Seed cultures were obtained from the American Type Culture Collection. The cells were grown in 10% FCS in DMEM supplemented with streptomycin (100 (μg/ml) and penicillin (100 IU/ml) and the medium was changed as required. Stock cultures were closely monitored and not allowed to grow to confluency prior to subculture. Test cultures were seeded so that cells were used just prior to, or at confluency.

**Human Skin Keratinocyte Culture** Primary cultures of human skin keratinocytes were prepared from neonatal foreskins. Tissue was obtained on the day of circumcision and incubated at 4°C for 24 hr in 25 U/ml dispase (Collaborative Research, Bedford, MA). The epidermis was removed and the cells segregated by a further 5 min incubation in 0.25% trypsin at 37°C. The trypsin was deactivated by the addition of 20% serum in medium and the cell suspension was centrifuged. The cell pellet was resuspended in Keratinocyte Serum Free Medium (KSFM, Gibco BRL, Grand Island, NY) and filtered through 70 μM nylon mesh. 75 $cm^2$ flasks were seeded at 5 x $10^5$ cells/8 ml KSFM supplemented with gentamicin (50 μg/ml) and Fungizone (0.25 μg/ml) and incubated in a 37°C humidified incubator in a 5% $CO_2$/95% air atmosphere. Cultures were re-fed every 2 - 4 days, as required. First passage cultures were seeded from log growth primary cultures at a density of 1000 cells/well in 96-well Costar multiwell plates, 10,000 cell/well in 24 multiwell plates, 50,000 cells/dish in 35 mm culture dishes, or at 2.5 x $10^5$ cells per 75 $cm^2$ culture flask. Apoptotic cell death was assessed in subconfluent cultures 16 hr after HD exposure.

**Chemical Treatment and Cytotoxicity Studies** On the day of chemical treatment the cultures were treated with freshly prepared treatment medium so that the desired final HD concentration was reached at 0.25% ethanol (v/v). The viability of HD-exposed cultures was determined 24 hr after exposure. To assess cytotoxicity, alamarBlue (AccuMed International Inc., Westlake, OH) was added (10%, v/v) and the cultures were allowed to incubate with the dye for the last 2 – 3 hr of the treatment time period. The absorbences

(570 nm - 600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). For the assessment of BzATP toxicity, CHO-K1 cells were treated with the drug dissolved in defined sucrose buffer (see below) for varying time intervals. 24 hours later cell viability was assessed using either the alamarBlue assay or LDH release. The 24 hr toxicity of maitotoxin (in culture medium) was also assessed in CHO-K1 cells using alamarBlue. Median lethal concentration ($LC_{50}$) values were determined graphically from experiments utilizing 6 wells per data point. Sulphur mustard was prepared by the Chemical Biological Defence Section, Defence Research Establishment Suffield at greater than 99% purity.

**Apoptosis Detection Techniques**

**Genomic DNA Analysis** Test cell samples were lysed in 50 µl of lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, and 1% Nonidet P-40) for 30 sec. After centrifugation at 2,000x*g* for 5 min at room temperature, the fragmented DNA in the supernatant was collected. After repeating the centrifugation step once, the supernatant was further treated at 56°C for 2 hr with RNase A (5 mg/ml) and SDS (1%), followed by digestion with proteinase K (2.5 mg/ml) at 37°C for 2 hr. DNA was precipitated and electrophoresed in 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and visualized under UV light. A 100-bp DNA ladder (Bio-Rad, Mississauga, Ontario, Canada) was used as a size marker.

**TUNEL Reaction** For *in situ* nick end labeling (TUNEL reaction) cells were plated onto 25 mm Thermonox plastic culture coverslips and allowed to grow to subconfluency prior to experimental use. After HD treatment the medium was removed and the coverslips were washed twice with PBS, and then fixed with 4% paraformaldehyde for 30 min. The cells were then permeabilized for 2 min with 0.1% triton X-100, 0.1% sodium citrate and after air drying, 50 µl of TUNEL reaction mixture (Roche Molecular Biochemicals, Laval, Quebec, Canada) was added so as to cover all cells. The coverslips were incubated at 37°C in a humidified chamber for 60 min and then rinsed 3 times with PBS prior to analysis by fluorescence microscopy.

**Soluble DNA** Soluble DNA was measured by the method described by Cui *et al.* (1994) with modifications. The cells were grown in 24 well plates and log phase growth cultures were radiolabeled by incubation with [$^3$H]thymidine (1 μCi/ml, Amersham Canada Ltd.,Oakville, Ont, Canada) overnight at 37°C in a humidified 5% $CO_2$ incubator. The medium containing [$^3$H]thymidine was then removed and the cells rinsed once with PBS. After drug treatment, aliquots of the culture medium (1ml/well, part A) were saved for radioactivity measurement and the cells were lysed in 0.5 ml TET (10 mM TRIS-HCL pH 7.5, 2 mM EDTA, 0.2 % Triton X-100) at 4°C for 30 min. The cell-lysate was then centrifuged (22 min, 14,000x*g*) and the resulting supernatant (part B) was removed and counted. The lysate pellet (part C) was solublilized with 1N NaOH (0.3 ml/microfuge tube) and counted. The experiments were performed in triplicate. Soluble DNA (percentage) was calculated according to the following formula: (Part A (dpm) + Part B (dpm))/ (Part A (dpm + Part B (dpm) + Part C (dpm)) x 100.

**Morphological Observations** Cell cultures were grown to subconfluency in 24 well plates prior to experimentation. After HD treatment, the cells were washed with PBS and then stained with 10 μl dye mix (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS) as previously described (Duke and Cohen, 1992). The cells were then visualized and scored using fluorescence microscopy. A minimum of 200 cells was visualized and the incidence of each of the following four cellular states was recorded; i) viable cells with normal nuclei (VN; bright green chromatin with organized structure), ii) viable cells with apoptotic nuclei (VA: bright green chromatin which is highly condensed or fragmented), iii) nonviable cells with normal nuclei (NVN; bright orange chromatin with organized structure) and iv) nonviable cells with apoptotic nuclei (NVA; bright orange chromatin which is highly condensed or fragmented). The percentages of apoptotic and necrotic cells were then calculated according to the formula:

% apoptotic cells = (VA+NVA)/(VN+VA+NVN+NVA) x 100.

% necrotic cells = NVN/(VN+VA+NVN+NVA) x 100.

**P2X Receptor Studies**

**Immunohistochemistry** Protocols for immunohistochemical staining were obtained

from Michael Iagallo (Histopathology Service Lab, UBC, personal communication, 1996). Rat tissues (synaptosomes and brain) were collected, rinsed in PBS, and fixed in 4% paraformaldehyde for 16 hr at 4°C. Tissues were then placed in PBS followed by PBS/10% sucrose (as a cryoprotectant) and frozen in OCT compound. Cryostat sections were cut at 8 μm and collected on adhesive coated microscope slides. Slides were then placed in PBS/0.01% Triton X-100, warmed to 37°C for 5 min to remove OCT compound, covered with Sequenza cover plates (Shandon, Pittsburgh, PA) and placed in a humidified Sequenza slide rack. Primary antibodies (anti-P2X1, anti-P2X2, anti-P2X4, and anti- $P2X_7$, Alomone Labs, Jerusalem, Israel) were applied and incubated for 16 hr at room temperature. Slides were rinsed three times with PBS/Triton X-100. Secondary antibody (Oregon Green 488-labelled goat anti-rabbit, Molecular Probes, Eugene, OR) was applied to sections for 2 hr under the same conditions. Following incubation, the slides were washed in three changes of PBS/Triton X-100, and coverslipped using Prolong anti-fade mounting media. A negative procedural control, in which the primary antibody was omitted and replaced with diluent buffer only, was used with each series. After the final PBS rinse, the slides were mounted using Prolong anti-fade mounting media (Molecular Probe) and coverslipped. Slides were visualized using fluorescence microscopy and representative images taken with a Spot 2 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

**Synaptosome Preparation** Synaptosomes were prepared using the Percoll® gradient method (Dunkley *et al.,* 1988). Briefly, the $S_1$ sucrose/EDTA supernatant was layered over a discontinuous Percoll® (Sigma, St Louis, MO) gradient consisting of 2 ml each of 3, 10, 15 and 23% (v/v) Percoll® dissolved in 0.32M sucrose containing 100 mg $L^{-1}$ EDTA. This was centrifuged at 20,000x*g* for 5 min at 4°C in a Beckman preparative centrifuge. The 10/15% and 15/23% interfaces were combined and diluted four-fold with Hanks balanced salt solution (HBSS) at pH 7.3, and then centrifuged at 12,500x*g* for 25 min. The resulting pellet was resuspended in HBSS. Aliquots of the Percoll® purified or crude $P_2$ synaptosomes were deposited on glass microscope slides (500 μl, Cytospin) and used for fluorescence histochemistry (outlined below). The composition of HBSS was as

follows in mM: KCl 5.4, $KH_2PO_4$ 0.5, NaCl 136, $NaHPO_4.7H_20$, 0.34, D-glucose 5.6 and $CaCl_2$.

**Western-Blotting** Rat brain, or purified rat synaptosomes were mixed and incubated on ice for 20 min with 300-500 µl 1 x SDS gel loading buffer, containing 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol. Protein concentration was measured using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). The sample was heated in boiling water for 5 min and an equal amount of protein from each sample was electrophoresed on 8% SDS acrylamide gels and electrophoretically transferred to a nitrocellulose membrane. Prestained SDS-Page standards (Bio-Rad, Mississauga, Ontario, Canada) were used to ensure the successful transfer and measure the size of any signal thereafter. The membrane was blocked with PBS containing 0.1% Tween (PBST) and 5% skim milk overnight at 4°C and then washed for 10 min with PBST. This step was repeated an additional three times. The washed membrane was incubated with a 1:300 dilution of P2X1, $P2X_2$, or $P2X_7$ antibody in PBST solution for 90 min, washed again and incubated for 90 min with a peroxidase labeled anti-rabbit antibody (1:3000 dilution, included in ECL-Kit from Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The membrane was washed again and positive bands were visualized with the enhanced chemiluminescence reagents following the instructions of the manufacturer. $P2X_1$ and $P2X_2$ antibodies were obtained from Alomone Labs (Jerusalem, Israel) while $P2X_7$ antibodies were obtained from two different sources (Alomone Labs, Jerusalem, Israel and Chemicon International, Inc., Temecula, CA), with similar results being obtained using both antibody sources.

**Cytolytic Pore Studies**

**Buffer Solutions** Physiological ionic buffer was made with the following composition in mM: KCl, 5; $MgSO_4$, 1; $CaCl_2$, 1; $Na_2HPO_4$, 1; D-glucose, 5.5; $NaHCO_3$, 5 and HEPES 20 (pH 7.4). *Sucrose buffer*: This low ionic strength medium was similar to that described by Michel *et al.,* (1996), and Surprenant *et al.,* (1996) in order to maintain lower concentrations of certain interfering ions. It consisted of the following in mM:

sucrose 280; $CaCl_2$ 0.5; KCl 5, D-Glucose 10, HEPES 10, N-methyl-D-glucamine 5, with the pH adjusted to 7.4.

**Ethidium Bromide Uptake Studies** CHO-K1 cells were allowed to grow until just confluent. The cells were gently trypsinized at room temperature and pelleted by centrifuging at 1000x*g* for 5 min. The pellet was suspended at a density of $10^6$ cells/ml in 20 μM ethidium bromide dissolved in either physiological ionic buffer (Mutini *et al.*, 1999) or sucrose buffer. The cell suspensions were protected from light and kept on ice until used. At the time of experimentation 2 ml of cell suspension were placed into a quartz cuvette and fluorescence (excitation: 360nm/emmision: 580 nm) was acquired for 5 min prior to drug treatment and for at least 30 minutes afterwards. Confirmatory evidence of ethidium bromide uptake was obtained by visualizing the cells using fluorescence microscopy. Measurements were made with reference to a 100 % response induced by digitonin.

**Ionic and pH Studies**

**Buffers** All buffers used in the ionic studies were isoosmotic with F-12 culture medium, which has a NaCl concentration of 130 mM and is 300 milliosmolal. Buffer composition consisted of 10 mM glucose, 10 mM HEPES, the test salt and sucrose so that the final solution was 300 milliosmolal, pH 7.4. The buffers were filter sterilized.

**HD Exposures** In studies that examined the effect of pH and ionic environment on the toxicity of HD, confluent cultures of CHO-K1 cells were used. Just prior to HD exposure the cultures were aspirated and the medium replaced with freshly pH'd medium. For ion studies, the cultures were rinsed twice with the test ion buffer before HD exposure. Immediately following these steps, the cells were exposed to HD dissolved in the test pH or ion buffer. The exposure was carried out for 1 hr at 37 °C, and then the cultures were changed into normal culture medium. For those experiments looking at the effects of pH and ion concentration on metabolic viability, toxicity was assessed 24 hr post-exposure using the alamarBlue assay. For studies examining the effects of pH on apoptotic cell death, DNA fragmentation and morphology was assessed 5 hr post-exposure.

## ROLE OF $P2X_7$ RECEPTORS IN HD AND ATP CYTOTOXICITY


### SUMMARY

Last year's report detailed the difficulties we encountered in documenting ATP induced cell death. However, a specific inhibitor of the $P2X_7$ receptor, oATP, was shown to have some interesting effects on HD-induced cell death. We have supplemented these findings and in this report show definitively that oATP potently inhibits the HD-induced DNA fragmentation that has become one of the hallmarks of apoptotic cell death. However, this does not result in a reduction of HD cytotoxicity, since the cells shunt from a primarily apoptotic cell death to one that is predominantly necrotic in nature. We have also been able to show, by minimizing the ionic strength of the incubation buffers, that BzATP, a potent analogue of ATP, was toxic in a concentration-dependent manner, and that this toxicity required only very limited exposure times in order for toxicity to be maximal. Furthermore, oATP was extremely effective in preventing BzATP-induced toxicity, confirming the mechanism of action as taking place at the level of the $P2X_7$ receptor.


### RESULTS

**Effects of oATP** oATP is a selective inhibitor of the $P2X_7$ receptor (Murgia *et al.*, 1993). Figure 1 shows the effect of a 2 hr pre-incubation of 300 or 500 μM oATP on HD-induced DNA fragmentation as assessed using gel electrophoresis. DNA from control cultures shows no fragmentation, while DNA isolated from CHO-K1 cells exposed to HD has fragmented into a "ladder" pattern. Preincubation with both oATP concentrations clearly inhibits the DNA fragmentation. This phenomenon is also shown in Figure 2, where DNA fragmentation is quantitated using the TUNEL reaction. Vehicle control or oATP treatment only, gave limited TUNEL positive cells. In contrast, HD increased the percentage of TUNEL positive cells in a concentration-dependent fashion. Pretreatment with either concentration of oATP (2 hr), dramatically decreased the incidence of TUNEL positive cells. Figure 3 depicts the effects of oATP on the morphological changes induced by HD in CHO-K1 cells. HD induced a concentration-dependent increase in total cell death, as well as apoptotic cell death. The incidence of

necrotic cell death was incidental compared to apoptosis, and did not appear to be as concentration related. Pretreatment with oATP did not reduce cell death, but clearly and dramatically decreased apoptosis and caused necrosis to become the predominant mode of cell death. This explains the results shown in Fig. 4, which clearly show that oATP did not have any effect on overall HD toxicity, as assessed using metabolic viability (alamarBlue) at 24 hr.

**BZATP Induced Cell Death** Experiments were carried out to determine the effects of ionic composition on BzATP cytotoxicity in CHOK-1 cells by utilizing a buffer which contained a low ionic concentration and using sucrose to adjust the buffer to physiological osmolality (Michel *et al.*,1996; Surprenant *et al.*, 1996; outlined in Methods). The results presented in Fig. 5 reveal that as the concentration of BzATP in sucrose based buffer was increased above a threshold toxic dose of about 5 μM, there was a sharp increase in cell death that was somewhat dependant on the length of incubation time, but nevertheless occurred extremely quickly. BzATP induced cell toxicity appeared to reach maximal values following about 30 min of incubation for any given concentration of BzATP examined. The relative lack of BzATP toxicity in routine culture medium is shown for contrast. The toxicity of BzATP in the sucrose based buffer was rather remarkable in relation to the lack of effect of BzATP in culture medium as shown here, as well as in last year's report (see Fig. 23), where toxicity was still absent at 500 μM in studies carried out in a normal physiologically based buffer system.

Figure 6 shows the protective effects of oATP pretreatment on BzATP induced cytotoxicity. The toxicity of BzATP was reversed in a concentration-dependant manner with concentrations of 100 μM oATP or larger. We are currently examining whether $P2X_7$ activation leads to apoptosis or necrosis or both types of cell death. BzATP induced cell death was also confirmed by light microscopic examination of BzATP exposed cells.

## DISCUSSION

The results of this study were directed toward understanding the mechanism underlying the initiation of BzATP induced toxicity in CHO-K1 cells so that studies on

the cytotoxic effects of HD could then be examined. This cell type was chosen in part due to their complement of $P2X_7$ receptors exclusive of other P2X subtypes, and in part due to the fact that their pharmacology has been previously well studied (Michel *et al.*, 1998). These cells therefore provided an ideal model with which to study the role of the $P2X_7$ receptor as it relates to HD toxicity. ATP and its analogues, particularly BzATP, are broad-spectrum cytotoxic agents that share a number of similarities with HD with respect to activation of cell death. For example, both HD (last report, present report, Papirmeister *et al.*, 1991) and ATP analogues kill cells and promote apoptosis and/or osmotic lysis (necrosis). ATP analogues cause cytotoxicity through activation of distinct cell surface $P_2$ purinoceptors (Di Virgilio e*t al.*, 1995; Burnstock, 1998). Some of these receptors, notably the P2X sub-family, are ionotropic channels, acting for example, to transport calcium through the cell membrane (Burnstock, 1998), resulting in a variety of physiological effector responses, such as muscle contraction or nerve transmission. At least two of the ATP receptors in this sub-family have been implicated in the mediation of ATP-induced cell death. The $P2X_1$ receptor, in addition to acting as a cation gated pore, has been shown to be upregulated in cells undergoing apoptosis (Chvatchko *et al.*,1996; Zambon *et al.*, 1994). This particular receptor has marked sequence homology (Valera *et al.*,1994; Brake *et al.*,1994) to the product of the gene known as RP-2, which has been shown to encode for apoptosis (Owens *et al.*,1991). Activation of the $P2X_7$ receptor also initially leads to the opening of an ionic channel, which in turn causes the opening of a membrane pore. This pore, in its open state permeabilizes the membrane to a variety of larger molecular species (up to about 900 daltons, Wiley *et al.*,1996). The literature suggests that activation of the $P2X_7$ receptor initiates caspase induction, leads to PARP cleavage, DNA fragmentation, release of cytokines, the activation of phospholipases and results in apoptosis and under certain conditions also necrosis (Di Virgilio 1995; Zheng *et al.*, 1991; Ferrari *et al.*, 1999; Coutinho-Silva *et al.*, 1999; Schulze-Lohoff *et al.*, 1998; Humphreys and Dubyak 1996; Humphreys *et al.*, 2000). Obviously, the effects of $P2X_7$ receptor activation mimic many of the effects of HD, including the initiation of cell death by both cytotoxic mechanisms.

**BzATP Induced Cytotoxicity: Role of Ions** One of the major difficulties encountered

in our studies (last year's report) was our inability to demonstrate cell death following $P2X_7$ activation in CHO-K1 cells. Since the dependency of $P2X_7$ receptor association with its agonists had been previously demonstrated to be partially related to ionic composition of the incubation medium (Michel *et al.,* 1996; 1999, Surprenant *et al.,* 1996; Virginio *et al.,* 1997), studies were initiated to observe the effects of ionic composition on BzATP induced toxicity. To our surprise we found that the toxic effects of $P2X_7$ receptor activation were directly related to the ion composition in the media. Furthermore, this dependence was much more important than generally indicated in the literature. A considerable effort has therefore been devoted to the examination of ionic effects on receptor activity as it relates to cell death. These results explain our earlier inability to demonstrate the toxicity of BzATP (or ATP) in the ion based buffers which were used in our earlier work. These results also led to studies into the effect of ionic environment on HD toxicity. This is also the reason that many of the experiments have been carried out in two buffer systems since it was not possible to determine prior to the experiments which buffer would be more suitable.

**$P2X_7$ Inhibition: Effect on HD Toxicity Profile** oATP effectively inhibited the DNA fragmentation and apoptotic morphological changes typically induced by exposure of CHO-K1 cells to HD. The $P2X_7$ inhibitory activity of oATP was confirmed by its ability to inhibit BzATP-induced cytotoxicity, as well as BzATP-induced ethidium uptake. However, despite the inhibitory effects of oATP on HD induced apoptosis, it was also clear that the overall toxicity of HD was unaffected. These results suggested that the cells that had originally followed the apoptotic pathway underwent cell death through a different mechanism following the blockade of the $P2X_7$ receptor. This, in fact, was confirmed by our morphological data, that clearly showed that the pathway to cell death became predominantly necrotic in nature when the cells were incubated with oATP prior to HD exposure. A number of recent studies have demonstrated a link between $P2X_7$ receptor activation and initiation of the biochemical cascade leading to apoptosis (Di Virgilio 1995; Zheng *et al.*, 1991; Ferrari *et al.*, 1999; Coutinho-Silva *et al.*, 1999; Schulze-Lohoff *et al.*, 1998). In addition, several studies have now identified situations where a shift from apoptosis to necrosis has occurred following prolonged stimulation of

the $P2X_7$ receptor or in situations where apoptotic stimulae have been impeded. Other recent studies have also identified the simultaneous development of both necrotic and apoptotic features following $P2X_7$ activation (Schulze-Lohoff *et al.*, 1998; Ferrari *et al.*, 1999).

Our studies thus appear to support a role for the $P2X_7$ receptor in HD-induced cell death. Under normal circumstances, HD induces a primarily apoptotic cell death through actions at the $P2X_7$ receptor (and probably other sites). However, when this receptor is blocked (ie. with oATP), the pathway is shunted to one that is primarily necrotic in nature and no apparent change is observed in the overall cell death observed. In the next section we hypothesize that some toxicants may be mediated by the dilation of a pore that allows larger molecular weight species into the intracellular space, resulting in cell death. This pore is mediated by at least two receptors, one being the $P2X_7$ receptor whose activation results primarily in apoptotic cell death, as well as some necrosis. The other is a receptor whose activation results primarily in a necrotic cell death and whose prototypical agonist is maitotoxin. It may be that $P2X_7$ inhibition by oATP shunts HD induced cell death to one that is primarily determined by the maitotoxin activated side of the pore. This would result in a diminished apoptotic death and at the same time an increase in necrotic cell death. Studies will be directed towards this possibility.

## ROLE OF A CYTOLYTIC PORE IN HD TOXICITY

### SUMMARY

During experiments designed to elucidate any possible interactions between HD and $P2X_7$ activation, it came to our attention that another agonist/receptor/pore complex, defined by maitotoxin, might be closely associated with the BzATP receptor pore. Subsequent studies characterizing this pore have revealed several new findings and show that a cytolytic pore exists in CHO-K1 cells (and presumably other cells) and that its dilation (and subsequent cytolytic activity) is dependent on two different receptors having unique characteristics. Activation of the $P2X_7$ receptor causes a dilation of the pore that is characterized by an influx of large molecules into the cell. This activation is independent of extracellular calcium and is inhibited by the presence of monovalent cations. The other receptor is activated by concentrations of maitotoxin that are much lower than BzATP – which presumably accounts for maitotoxin's much greater potency/toxicity than BzATP (~ 50,000 times). In contrast to the action of BzATP on the pore, maitotoxin is more effective in buffer containing monovalent ions, and its action is completely dependent on the presence of extracellular calcium. Preliminary evidence also appears to indicate that activation of this pore complex may result in differential modes of cell death depending on which side of it is activated; apoptosis and necrosis following $P2X_7$ activation, or necrosis following maitotoxin stimulation (see Fig. 7). Early work has also indicated that HD has some effect on the activity of this common cytolytic pore by modulating maitotoxin stimuli. These results offer strong evidence for the existence of a common cytolytic pore activated by different agonists and also suggests that other cytotoxic agents may activate this same pore. Future work will further investigate how HD acts at this site.

### RESULTS

**BzATP** Figure 8 depicts ethidium bromide uptake in CHO-K1 cells following activation of the $P2X_7$ receptor by BzATP. Cell suspensions were incubated at 37°C in SBB containing 20 μM ethidium bromide. The cells were then exposed to BzATP (100 μM) and the uptake of the dye followed fluorimetrically (Panel D). In addition, samples of the

cell suspension were removed from the cuvette prior to (Panel A), and 15 min after (Panel B) BzATP exposure and visualized using fluorescence microscopy. In contrast to non-treated control samples, where no increase in fluorescence was observed (baseline, Panel D), BzATP induced a gradual, time related increase in fluorescence. Confirmation of these observations was obtained using microscopy, where dye uptake was obvious in BzATP treated cells (Panel B), but not in control cells (Panel A). The percentage of BzATP treated cells fluorescing is near 100 %, as evidenced by imaging of the cells pictured in Panel B with light microscopy (Panel C). All experiments were carried out using similar cell densities and therefore light micrographs of test cell suspensions will not be routinely included in subsequent figures. Figure 9 shows typical responses obtained with different concentrations of BzATP. The response is almost maximal by 50 μM, and definitively maximal by 100 μM BzATP.

Figure 10 shows the effect of incubating CHO-K1 cell suspensions with the $P2X_7$ inhibitor, oATP (300 μM) for 30 min prior to BzATP treatment. 100 μM BzATP induces dye uptake (Panel A). However, oATP completely blocks this uptake and no cells fluoresce as assessed using microscopy (PanelB) or fluorimetry (PanelC).

The effects of ionic environment on BzATP induced ethidium bromide uptake are illustrated in Figures 11 and 12. 200 μM BzATP induced little or no dye uptake in normal physiological buffer (Fig. 11, Panel B). However, when the experiments were carried out in buffer with significantly decreased ionic content (but the same calcium concentration), a definite time-dependent increase in fluorescence was observed (Fig. 11, Panel B). In contrast, calcium appeared to have little or no role in opening the pore; dye uptake was similar when BzATP exposure was carried out in buffer with, or without 0.5 mM calcium (Fig. 12, Panel B).

**Maitotoxin** In order to characterize maitotoxin induced opening of the pore, similar experiments were carried out to those performed with BzATP. Figure 13 shows the potent effects of maitotoxin on pore opening as measured using either fluorescent microscopy of fluorimetry. Panel A shows that non-treated control cells did not fluoresce. However, treatment with 1.0 nM maitotoxin induced a rapid and robust increase in dye uptake that resulted in the cells fluorescing intensely (Panel B). However,

this maitotoxin concentration was not optimal in inducing dye uptake. As shown in Fig. 14, greater responses were observed with 3.0 and 6.0 nM maitotoxin (Panel B). Figure 14A shows an average dye uptake in the two buffers. Figure 15 shows that the results obtained with maitotoxin induced ethidium bromide uptake, parallel those obtained when assessing the viability of the cells 24 hr post-treatment using the metabolic viability indicator, alamarBlue. The $LC_{50}$ obtained using this assay was 433 pM +/- 21 pM (n = 3), showing that the maitotoxin concentrations used to induce dye uptake were indeed *toxic* concentrations. A direct comparison of the effects of BzATP and maitotoxin on ethidium bromide uptake is presented in Fig.16, which shows typical dye uptake responses during a 1 hr period of exposure to maitotoxin (3.0 nM -A) or BzATP (200 μM -B) in buffers that maximize their activity. This figure illustrates that these agonists induced similar qualitative, but not quantitative dye uptake and that the pore activation was progressive with time. Both responses also appeared to be biphasic in nature.

In order to determine whether the maitotoxin induced dye influx was the result of $P2X_7$ receptor stimulation, as was the case for BzATP, cells were preincubated with oATP prior to maitotoxin exposure. Panel A of Fig. 17 shows the presence of numerous fluorescent cells following a 15 min exposure to 1.0 nM maitotoxin. Pretreatment with 300 μM oATP had no effect on maitotoxin induced ethidium bromide uptake (Panel B or C).

Figure 18 demonstrates the typical effects of buffer composition on maitotoxin receptor stimulation and ethidium bromide uptake in CHOK-1 cells. The left hand panel (A) shows uptake over a 30 min period in cells exposed to 3.0 nM maitotoxin and incubated in sucrose based buffer, while the right hand panel (B) illustrates the response in PSS. The response in sucrose buffer failed to reach maximal values and is about 50 % of maximal following 30 min. In PSS the same concentration reached 100 % of the maximally attainable response in about 25 min. This dependence of induced dye uptake on the ionic composition of the buffer is also illustrated in Fig. 14. In panel (A), a time related effect of 1.0 nM maitotoxin in sucrose or in PSS is seen after a 10, 20 or 30 min exposure. It is also clear that the response is considerably greater in the PSS.

Maitotoxin induced dye uptake was very sensitive to the presence of extracellular calcium, as shown in Fig. 19, which depicts a representative experiment examining the

effect of external calcium concentration on 1.0 nM maitotoxin induced ethidium bromide uptake in CHO-K1 cells. Maitotoxin produced no ethidium bromide uptake in buffer to which no calcium was added. However, increasing the calcium concentration resulted in a concentration dependent increase in dye uptake.

**Sulphur Mustard** Figures 20 and 21 reveal the effects of HD on ethidium bromide influx in CHOK-1 cells. Cells were incubated with 400 μM HD for one hour and dye uptake was followed using both fluorimetry (Fig. 20) and microscopy (Fig. 21). In neither case did HD cause an appreciable increase in fluorescence. Figures 22 and 23 show the effect of a 10 minute, 400 μM HD pretreatment on BzATP induced ethidium bromide uptake. It is obvious that there was no effect on dye uptake. These results are currently being analyzed for fluorescent intensity by computer aided techniques.

## DISCUSSION

**Cytolytic Pore Receptor Complex** During the experiments concerning the interactions of BzATP and HD, it came to our attention that there appeared to be another receptor pore complex which might be closely associated with the BzATP receptor activated pore. This receptor appeared to be also linked to a cytolytic pore and maitotoxin (an exquisitely toxic compound derived from marine organisms), was the prototypical agonist in this second complex (Schilling *et al.*, 1999a,b). This fact opened new avenues for investigation, since it appeared likely that there was a larger, more pervasive cytolytic complex, consisting possibly of two receptors and two pores, which might serve as sites for interaction with HD. This posed the possibility that if HD did produce its cytotoxic effects by activating the cytolytic pore, it could do so either through the $P2X_7$ "side" of the pore or the maitotoxin "side", or directly on the pore. We therefore examined the interaction of these sites with each other and HD. These studies have led to some exciting new information concerning the mechanisms of cell death in CHO-K1 cells, and perhaps in other cell types that contain these receptors. At the present time we feel that these studies (although incomplete) provide reasonable evidence that CHO-K1 cells contain a site in the membrane that consists of two receptors; a maitotoxin receptor and a $P2X_7$ receptor that separately control a membrane pore (Fig. 7). The results presented

here provide evidence that the cytolytic pore was common to both receptors, but activated by two very different set of stimulae. Perhaps the most definitive evidence that the pore was controlled by two different mechanisms was that the $P2X_7$ mediated, BzATP induced dye uptake, was inhibited by oATP, while that of maitotoxin was unaffected. In addition, the two agonists had a totally different profile with respect to their activity in ionic medium. BzATP failed to induce ethidium bromide uptake (or cell death) in ion based buffers, while maitotoxin's were enhanced in these same ionic buffers. This was intriguing, since we also report the very significant role that the ionic environment plays in HD toxicity (described in another section of this report). Another factor suggesting that pore activation was activated at separate sites was the difference in potencies of the two agonists. Maitotoxin was clearly active at 100 pM and induced a reasonably steep concentration response curve, while BzATP, which is the most active agonist known at the $P2X_7$ receptor, caused dye influx at a threshold concentration of only 50 μM, with a relatively shallow concentration response curve.

Maitotoxin induced ethidium bromide uptake and cell death were totally dependent on the influx of calcium from the external media (present study, see also Zhao *et al.,* 1999; Kutty *et al.,* 1989) and the cell death appeared to occur totally through necrotic mechanisms (present report, see Zhao *et al.,* 1999). On the other hand, our results, although not complete at this time, indicate that BzATP stimulation of ethidium bromide influx was not significantly altered by external calcium concentrations. These results, if confirmed by further experiments, suggest the rather exciting prospect of the identification of a cytotoxic complex in the cell membrane that is activated, or modulated by very different external stimulae. Furthermore, this complex also appears to result in a totally different cellular response, apoptosis (possibly with necrosis) following $P2X_7$ stimulation, or necrosis following maitotoxin stimulation. It would be extremely interesting to examine the role of calcium or other ions on the different cellular responses following ethidium bromide influx or to further study the activity of caspases or calpain following pore activation through each mechanism. This pore might conceivably be a common site in the initiation of both cell death responses that could be modulated by quite distinct mechanisms. Table 1 shows a summary pointing out similarities and differences in mechanisms underlying the opening of the cytolytic pore.

TABLE 1

Studies on the Activation of the $P2X_7$/Maitotoxin Cytolytic Pore in CHO-K1 Cells

| $P2X_7$ Activated Pore | Maitotoxin Activated Pore |
|---|---|
| BzATP + certain P2X agonists | Unaffected by $P2X_7$ agonists |
| Inhibited by $P2X_7$ antagonists | Not affected by $P2X_7$ antagonists |
| Inhibited by certain undefined ions, less active in ionic buffers | More active in ionic buffer than in sucrose-based buffer |
| Uptake of ethidium bromide is time-dependent | Uptake of ethidium bromide is time-dependent |
| Ethidium bromide uptake demonstrates a very shallow concentration effect curve | Uptake of ethidium bromide is very concentration dependent |
| Stimulation results in cell death by apoptosis/necrosis | Stimulation results in cell death by necrosis |
| Receptor acts as an ionic channel. Subsequent pore opening is $Ca^{2+}$ independent | Receptor acts as ionic channel, $Ca^{2+}$ influx necessary for secondary pore opening |
| Stimulation releases inflammatory mediators | ? |
| Stimulation activates caspase 3 | ? |

**Effects of HD on the Common Cytolytic Pore** Studies were initiated to determine the effects of HD on the two receptor "sides " of the common cytolytic pore, measured by the uptake of ethidium bromide in CHOK-1 cells. In initial experiments, HD 100 μM, was incubated with CHOK-1 cells and ethidium bromide uptake was followed for up to 1 hr. Over this time period the dye uptake as assessed by kinetic analysis or fluorescence microscopy was either small or non-existant. HD treated cells observed by fluorescent microscopy appeared slightly more stained than the untreated cells. The slides are currently being examined with new software in order to quantitate the fluorescence.

In other experiments, cells were incubated with higher concentrations of HD (400 μM) in ethidium bromide solution for shorter periods of time and then exposed to BzATP 100 μM. No change in ethidium bromide uptake from BzATP only treated cells was observed. The lack of effect of HD on dye uptake appears at first sight to argue against any role of HD on what we have termed here as the cytolytic pore. However, it must be considered that HD induced cellular responses begin shortly after exposure but no visual cell death can be seen for many hours. Both maitotoxin and BzATP, on the other hand, initiate ethidium bromide uptake and cytotoxic cell responses relatively rapidly.

**Physiological Role of Ions in Cell Death** One of the major roles of extracellular ATP appears in states where high concentrations occur in the cellular environment as the result of cell injury. In particular, ATP is released from cells that are ischemic or anoxic – where concentrations can reach mM levels. Not only are the cells exposed to maximal extracellular concentrations of ATP, but under these conditions it has been reported that ionic gradients across membranes "collapse". It is believed therefore, that the collapsing gradients in the presence of elevated concentrations of ATP would favor the activation of cytotolytic $P2X_7$ receptors initiating cell death. Thus the cytolytic pore may become and remain activated for long periods of time.

Experiments to date have revealed that both BzATP and maitotoxin caused concentration-dependent activation of a membrane pore that leads to uptake of the fluorescent dye, ethidium bromide. The uptake of this dye is usually an accurate reflection that the cell will ultimately undergo cell death. The activity of the pore following the stimulation of the $P2X_7$ receptor has been related to the initiation of

cytotoxicity through both apoptosis and necrosis. The measurement of BzATP-induced cell death by LDH release were consistent with the results found with respect to the activation of the cytolytic pore reflected in the ethidium bromide uptake experiments.

The results indicate that BzATP opens the cytolytic pore primarily through activation of the $P2X_7$ receptor. Maitotoxin, on the other hand, clearly acts to open the pore through a different mechanism. The result appears in either case to be cell death, supporting a wider role for the cytolytic pore in the mediation of cytotoxicity. The cell death type (apoptosis/necrosis) with either of these cytotoxic agents is currently being evaluated.

We are currently examining the role of HD alone and possible interactions of HD with the pore itself or either the $P2X_7$ or the maitotoxin receptor. To date, the addition of HD to the cells for short periods of time provides some evidence that HD may induce minimal amounts of ethidium uptake. Again, initial results also indicate that addition of HD to CHO-K1 cells 10 minutes prior to a small (just above threshold) concentration of maitotoxin had little effect on maitotoxin-induced ethidium bromide influx. Interactions of the two agents on cell death are also planned. However, initial results using significantly higher concentrations of maitotoxin, in addition to HD, appear to suggest a significant potentiation of the maitotoxin-induced ethidium bromide uptake by HD pre-incubation. If this result can be substantiated, it would suggest a major conformational change of the maitotoxin receptor or cytolytic pore induced by HD. This may cause increased uptake of small molecular weight substances and alteration of cell contents leading to cell death.

These results have clearly delineated the existence of a cytolytic membrane pore that is permeable to small molecular weight molecules that may lead to cytotoxicity. The results also clearly show that this cytolytic pore is common to several different cytotoxic agents, but can be activated in a distinct manner. Specifically, these results also offer support for the contention that the $P2X_7$-activated cytolytic pore and the maitotoxin-activated cytolytic pore are the same pore, but are certainly not activated by the same mechanism. Activation of the $P2X_7$ receptor opens the pore but is sensitive to ionic composition and specific antagonists like oATP. The activation of what appears to be the same pore by maitotoxin is insensitive to ions and to $P2X_7$ blocking agents.

# THE ROLE OF CALCIUM IN SULPHUR MUSTARD INDUCED APOPTOTIC CELL DEATH

## SUMMARY

For several years now, the role of calcium in the aetiology of sulphur mustard induced cell death has been controversial. Although it now seems to be accepted that this CW agent causes a modest, but irreversible elevation of intracellular calcium in several different cell types, the importance of this calcium release to the overall viability of the exposed cell is not known with certainty. There is evidence in the literature that supports the contention that this phenomenon is an important causal step in the development of HD induced toxicity, as well as evidence that refutes this stance. During this past year we have generated experimental data that supports a compromise to these two seemingly disparate viewpoints. We have shown, using human skin keratinocytes, that HD causes apoptotic cell death and that the DNA fragmentation characteristic of this type of cell death is dependent on the intracellular calcium release induced by HD. However, inhibition of this intracellular calcium elevation does not reduce the toxicity of HD; it appears to shunt the mode of cell death to one that is primarily necrotic in nature. For several months of this past year, our supply of human skin tissue was interrupted and we have only recently regained our source. In the near future we will be conducting additional studies to more firmly establish the role of intracellular calcium in HD-induced cell death in keratinocytes.

## RESULTS

Figure 24a-c (also included in last year's report) depicts the results of cytotoxicity studies using just-confluent cultures of human keratinocytes exposed to HD. In these experiments the effects of various treatments that are known to modulate intracellular calcium on mustard toxicity were examined. The calcium chelator BAPTA-AM and the endoplasmic reticulum $Ca^{2+}$ ATPase inhibitor thapsigargin, as well as varying external calcium concentrations did not alter the toxicity of HD at 48 hr in any way. However, BAPTA-AM and particularly thapsigargin appeared to reduce DNA fragmentation (soluble DNA at 16 hr) at 200 μM HD (Fig. 25). The effects of calcium modulation on

HD toxicity were next assessed using morphological means (Fig. 26). At 200 μM, total cell death (~ 30%) is approximately equally divided between cells undergoing apoptotic and necrotic cell death. Concentrations of thapsigargin and BAPTA-AM that have previously been shown to eliminate HD induced elevation of intracellular calcium (Hamilton *et al.*, 1998; Sawyer and Hamilton, 2000) did not significantly alter the total cell death. However, there were indications that drug pretreatment altered the profile of cell death so that necrotic cell death became predominant, as compared to cells undergoing apoptosis. Similarly, incubation of the cells in medium containing nominal zero calcium concentration may also alter the pattern of cell death. These results have been obtained quite recently and have not been completely analyzed. However, we consistently obtain similar results – a drug induced decrease in DNA fragmentation and concomitant changes in the mode of cell death towards one being more necrotic in nature.

## DISCUSSION

A variety of hypotheses have been advanced over the last several decades in efforts to explain the vesicant action of HD (reviewed in Papirmeister *et al.*, 1991). One of the most recent has focused on the role of calcium in chemically induced toxicity and is based on a scheme put forward by Orrenius and coworkers (Orrenius, 1985; Orrenius and Nicotera, 1987; Orrenius *et al.*, 1988). They proposed that toxicants that bind thiol compounds not only deplete glutathione, but also inactivate a number of proteins, ie. $Ca^{2+}$ ATPases, that are responsible for calcium homeostasis in the cell. This combined insult is suggested to cause a sustained intracellular calcium elevation with resultant activation of endonucleases, phospholipases and proteases that lead to cell death. More recently, we have proposed that the toxic effects of HD may be mediated through ATP receptors, a group of receptors that help modulate the access of calcium to the interior of the cell – resulting in a variety of physiological effector responses, including the activation of a host of lytic enzymes.

Several different laboratories utilizing a variety of cell culture systems have examined the effect of HD (~ 100 μM - 1.0 mM) on cellular calcium levels and have come to varying conclusions as to the importance of calcium homeostasis in HD-induced cytotoxicity. The first laboratory to test the thiol-$Ca^{2+}$ hypothesis found that in mouse

fibroblast B77 cells, HD induced a modest, but immediate and sustained rise in intracellular calcium levels that was independent of external calcium concentrations (Hua *et al.*, 1993). However, follow-up work by Mol and coworkers were equivocal. In second passage cultures of human skin epidermal keratinocytes grown on 3T3 feeder-layers, they found little or no evidence of HD-induced intracellular calcium elevation and dismissed the small increases they detected as reflecting abnormal cellular physiology rather than acute toxicity (Mol, 1994; Mol and Smith, 1996). In contrast, intracellular calcium was consistently found to be elevated 2- 6 hr after HD exposure in a mouse neuroblastoma-rat glioma hybid cell line (NG108-15) by Ray and colleagues (Ray *et al.*, 1995), who also found a similar elevation in human epidermal keratinocytes (Ray *et al.*, 1994). This same group has gone on to report that the cell-permeant calcium chelator BAPTA-AM not only prevented this intracellular calcium elevation, but was also effective in preventing HD-induced cytotoxicity (Ray *et al.*, 1996; 1997; 1998). Recent studies in our laboratory (Hamilton *et al.*, 1998) have tended to confirm the initial findings of Hua *et al.* (1993). In first passage just-confluent cultures of neonatal human skin keratinocytes, it was found that HD does indeed cause an immediate, concentration-dependent and sustained elevation of intracellular calcium that appeared to originate from intracellular stores.

The findings of Ray and coworkers have been recently supplemented with additional work carried out using human skin keratinocytes (Rosenthal *et al.*, 1998). These studies demonstrated that HD causes the cleavage of DNA into the distinct "ladder patterns" of DNA fragments on electrophoresed agarose gels, that have become one of the hallmarks of apoptotic cell death. Furthermore, they showed that both BAPTA-AM and the calmodulin inhibitor W-7, inhibited the formation of these "ladders". We therefore carried out studies investigating the role of calcium in HD–induced apoptotic cell death.

To this point this laboratory has failed to consistently obtain HD induced DNA ladders on agarose gels from keratinocytes. The reasons for this are unclear. However, since we routinely obtain HD induced DNA ladders in a variety of other cell types, we assume that this is a technical problem, possibly due to keratin. We therefore assessed DNA fragmentation using alternative assay systems.

The soluble DNA assay measures DNA fragmentation during apoptotic cell death and provide quantitative estimates of damage. Using this technique, we have been able to demonstrate a concentration dependent increase in HD induced fragmentation in a variety of different cell types (last year's report). Furthermore, in agreement with Rosenthal *et al.* (1998), we have also been able to demonstrate that both thapsigargin and BAPTA-AM appear to inhibit HD induced DNA fragmentation. In contrast, adjusting the external calcium concentration had no effect, showing that the DNAse(s) responsible for the DNA cleavage were exclusively dependent on *intracellular* calcium levels. Although these results were confirmatory of those of Rosenthal *et al.* (1998), they seemed to contradict our earlier findings that indicated that elimination of HD induced intracellular calcium elevation did not affect the resultant toxicity. In order to further investigate this apparent disconnect, we investigated the effects of HD on human skin keratinocytes using morphological means. This technique utilizes dual dye labeling of cells to visualize the gross morphology of both the cell body and the chromatin. 200 μM HD induced significant increases in both apoptotic and necrotic cell death as compared to solvent treated controls. Pretreatment of these cultures with concentrations of either thapsigargin or BAPTA-AM that have been demonstrated to eliminate HD induced intracellular calcium elevation, appeared to change the proportion of apoptotic cell death compared to necrotic cell death, so that necrosis became predominant. The sum total of these effects resulted in no change in the total cell death induced by HD.

In summary, although these results are preliminary and will have to be supplemented with additional work, they suggest that HD causes cell death characterized by the DNA fragmentation and morphological changes characteristic of apoptotic cell death. These events are normally preceded by an elevation in intracellular free calcium levels arising from intracellular stores. This elevation is necessary for apoptotic DNA fragmentation, but not for cell death; inhibition of this calcium release merely shunts the mode of cell death into one that is primarily necrotic in nature, such that there is no net effect with respect to cell viability by 48 hr post-HD exposure. It is interesting that shunting from apoptotic to necrotic cell death has also been observed to occur following exposure to other compounds, notably those that block the $P2X_7$ receptor.

## THE ROLE OF IONIC ENVIRONMENT IN SULPHUR MUSTARD INDUCED CELL DEATH

**Summary**

The importance of ionic environment in the toxicity of ATP to cells in culture has been well documented in our previous reports. In routine tissue culture media, we were unable to demonstrate ATP toxicity, accept at very high concentrations. However, in buffers containing no sodium, but adjusted to physiological osmolality (~ 300 milliosmolal) with sucrose, ATP and its analogues were shown to be quite toxic. This was an integral part of our efforts to demonstrate that HD may exert its toxicity at the level of ATP receptors and thus, follow-up studies consisted of trying to characterize any interactions between ATP and HD. These have met with difficulty, since, to our surprise, HD was virtually non-toxic to cells in culture when the exposures were carried out in the non-ionic sucrose buffers so necessary for ATP toxicity to be expressed.

The interesting finding that HD was essentially non-toxic in ion-free buffer, prompted us to examine the role of ionic environment, including pH, on HD toxicity in CHO-K1 cell culture. We show here that HD toxicity is strongly dependent on the ionic environment during the first hour of treatment. The toxicity of HD is very much reduced when the exposure is carried out in non-ionic (sucrose) buffers. Furthermore, as the ionic strength is increased, for example with NaCl (and other salts), HD regains its toxicity compared to that obtained in routine culture medium. Similarly, HD exposures carried out in basic medium much reduce the resultant toxicity of this agent at 24 hr.

**RESULTS**

Figures 27 and 28b depict the effect of varying NaCl concentration on HD toxicity in CHO-K1 cells. Cultures were exposed to varying concentrations of HD in culture medium, or in defined sucrose buffers of increasing NaCl concentration for one hour. The cultures were then changed into culture medium and assayed for cell viability 23 hr post-HD exposure. The $LC_{50}$ for HD in culture medium was ~ 130 μM, in contrast to a value of > 800 μM when the treatment was carried out in defined sucrose buffer. As the buffer NaCl concentration was increased, the toxicity of HD also became greater, so that by ~ 65.0 mM NaCl, the $LC_{50}$ approximated that obtained in culture medium. The

median effective concentration for NaCl was 10.8 mM +/- 2.9 mM (n = 6) with respect to increasing the toxicity of HD from that obtained in non-ionic sucrose buffer to one approaching the $LC_{50}$ obtained in physiological F-12 culture medium. Similar studies were carried out in buffer solutions where chloride was kept constant and the cation was varied. In every case, the toxicity of HD increased with salt concentration (Fig. 28, 29). Lithium was approximately half as effective as NaCl in rendering HD toxic, while KCl, RbCl, CsCl and choline Cl were one third as effective (Table 2). Figure 30 shows the effect of varying pH on the toxicity of HD when the incubations were carried out in culture medium. When the cultures were exposed to HD at a pH close to that of physiological pH (pH 7.5), the $LC_{50}$ was 143.1 μM +/- 9.0 μM. HD was only slightly more toxic when the treatments were carried out in medium at acidic pH. In contrast, the toxicity of HD was dramatically reduced as the treatments were carried out in medium at pH greater than 8.5, so that at pH 9.5, HD was almost four times less toxic ($LC_{50}$ = 593.7 μM +/- 53.7 μM). Figures 31 and 32 depict the protective effect of basic pH on the toxicity of HD as assessed using assays of apoptotic cell death. Figure 31 shows the effect of HD on DNA fragmentation as measured using the soluble DNA assay. At pH 7.5 soluble DNA increase in an HD concentration-dependent fashion up until 600 μM HD and then falls precipitously at 800 μM, a pattern that is consistently obtained with this assay. However, the increase in HD-induced soluble DNA is significantly reduced at basic pH. Similar results are obtained when assessing cell death using morphological means (Fig. 32). At physiological pH, HD induces cell death predominantly through apoptosis, with a small proportion of necrosis observed. A basic pH, very little HD-induced cell death of any type is observed using this assay. This information has been obtained only recently and hence the lack of statistical analysis. We are currently supplementing these studies with further experiments

Table 2 The effect of salt concentration on HD toxicity in CHO-K1 cells.

| Test Salt | n | Median Effective Concentration |
|---|---|---|
| LiCl | 3 | 19.0 mM +/- 1.0 mM |
| NaCl | 6 | 10.8 mM +/- 2.9 mM |
| KCl | 3 | 30.5 mM +/- 5.1 mM |
| RbCl | 3 | 31.8 mM +/- 2.7 mM |
| CsCl | 3 | 26.9 mM +/- 0.7 mM |
| Choline Cl | 3 | 32.9 mM +/- 5.9 mM |

**DISCUSSION**

The effect of ionic environment on P2 receptor function has been extensively studied in recent years and it is now well established that the action of ATP at the P2 receptor is largely determined by the presence of monovalent cations. Thus, in human lymphocytes, the response to ATP is enhanced when NaCl is replaced by KCl, choline chloride or N-methyl-D-glucamine chloride (Wiley *et al.*, 1992), while in murine macrophages a similar increase in the ability of ATP to stimulate phospholipase D is seen when NaCl is replaced with KCl or LiCl (Dubyak and El-Moatassim , 1993). A more recent study (Michel *et al.*, 1999) in HEK293 cells examined the effect of cations, as well as pH on $P2X_7$ receptor function. They found that the potency of BzATP was 19 times higher in NaCl-free buffer than in 140 mM NaCl containing buffer. Replacement of the sodium ion with either potassium of choline decreased the effect of the chloride salt. They also found that receptor function was optimal at pH 7.5, declining at pH values below 6.5 or above 8.5.

Ironically, the effects of ionic environment on the toxicity of HD were diametrically opposed to those on P2 receptor function and resultant ATP potency/toxicity. Thus, in contrast to ATP, HD was virtually non-toxic in non-ionic buffer, and regained its toxicity (relative to that in culture medium) in a concentration-response fashion only as the NaCl concentration was increased. The chloride salt of

lithium was about half as effective in rendering HD toxic, while potassium, rubidium, cesium and choline salts were equipotent and approximately one third as effective. The ionic effects on HD toxicity do not appear to be related to P2 receptor function and at this point it is unclear as to what site they act at to modulate the toxicity of HD so profoundly. It is tempting to ascribe the ionic effects on HD toxicity to alterations in the rate of HD hydrolysis by the chloride ion. However, increasing chloride ion concentration should serve only to retard hydrolysis to the highly reactive cyclic ethylene sulphonium ion – the putative toxic species (Papirmeister *et al.*, 1991). The *total* quantity of this species generated over the one hour exposure period would probably not account for the differences in HD toxicity observed in different ionic solutions. Studies to investigate this possible explanation for the ionic effects on HD toxicity will be carried out during this next year.

The effects of varying pH on HD toxicity were also dramatic, and very different from those on ATP potency. In contrast to the pH dependency of ATP, where P2 receptor function is optimal at physiological pH and decreases at either acidic or basic pH, HD toxicity changed relatively modestly from pH 5.0 to pH 8.0 and then declined precipitously as the pH became more basic. This pH dependence is somewhat unusual from a physiological standpoint, and once again, it is tempting to speculate that modulating pH changes the hydrolysis rate of HD in solution. However, HD hydrolysis is independent of pH (Papirmeister *et al.*, 1991), making it unlikely that these observations are a result of decreased HD hydrolysis.

The above observations have only recently been acquired and their significance has yet to be ascertained. Certainly, the use of seemingly non-physiological buffers makes the interpretation of results problematic and the likelihood that we may be observing changes in HD toxicity due to solution chemistry greater. This possibility will need to be definitively ruled out during the next year, although very preliminary data (not included in this report) appears to support a biochemical/physiological explanation to the above findings. The use of these types of buffers has been well documented in ion channel research, although the justification for their use, especially with respect to P2 receptor function, has generally been that during conditions of ischemia, ionic gradients collapse and potassium, sodium, chloride and calcium levels are altered so that $P2X_7$

receptor function is enhanced. Clearly, during these conditions, HD toxicity would be lessoned. Perhaps this represents a protective mechanism against other (natural) toxicants that share some traits with HD. Alternatively, the use of such buffers may be enabling us to sensitively characterize the ionic requirements of an early HD induced event that is causal to HD toxicity. This information may, in the future, facilitate its identification.

## PRESENCE OF $P2X_7$ RECEPTORS ON PRESYNAPTIC NERVE ENDINGS

In the past two yearly reports, we have described experiments that have identified $P2X_7$ receptors on pre-synaptic nervous elements and examined the possibility that HD might interact with these receptors to interfere with neurotransmission. We submit in this report some additional information that completes this part of the work. One figure (Fig. 33) will replace two from a previous report, since it is a clearer immunohistochemical depiction of the presence of $P2X_7$ receptors than we have previously shown. We have also added further proof (Figure 33, Panels C & D) that the synaptosomal preparation we have been working with was not contaminated by microglia. This was accomplished by demonstrating that OX-42, a protein specific to microglia was not present in our preparations (Figure 33, Panel F).

## KEY RESEARCH ACCOMPLISHMENTS

Last year's report clearly demonstrated that HD caused apoptosis in a wide variety of cell types. We reported difficulty in relating these effects to ATP receptor activation because of our failure to demonstrate cytotoxicity following exposure of cells to $P2X_7$ agonists. This was reported as a major problem area. We have now been successful in overcoming these problems and have demonstrated that activation of $P2X_7$ receptors causes cell death, that the death is related to the concentration of certain ions in the incubation medium, and that it occurs through activation of a cytolytic pore in the membranes of cells containing the receptor.

We have identified not only that the $P2X_7$ receptor exists in CHO-K1 cells, but have also characterized the factors that control the diameter of the pore linked to this receptor. Our results also clearly show that there is an additional receptor which when activated, also leads to cell death, but apparently through a mechanism that differs from $P2X_7$ receptor activation. This second receptor is stimulated by maitotoxin.

Activation of the $P2X_7$ receptor, as well as the maitotoxin activated receptor, both caused the concentration dependent uptake of ethidium bromide which appears to be closely related to cell death. The response of the membrane cytolytic pore suggested that it was common to both receptors, but activated by different mechanisms and therefore controlled by completely different factors. This is an important step towards our understanding of the cytolytic pore, which is linked to the initiation of both apoptosis and necrosis.

Preliminary results suggest that HD modulates the effects of maitotoxin, suggesting a role for the cytolytic pore in HD toxicity.

Initial results from recent experiments suggest that the cellular ionic environment, including pH, plays a major role in the cytotoxicity of HD.

The $P2X_7$ receptor antagonist oATP blocked BzATP-induced cell death. Although it failed to block HD cytotoxicity, it altered the proportion of cells that died from apoptosis as opposed to necrosis significantly, suggesting an effect somewhere on the $P2X_7$ receptor.

Early experiments using human skin keratinocytes indicate that intracellular calcium is necessary for the HD induced DNA fragmentation characteristic of apoptotic cell death. However, inhibition of HD-induced intracellular calcium mobilization merely shunts the mode of cell death to one that is predominantly necrotic in nature, and does not alter overall cell death.

## REPORTABLE OUTCOMES

Gong, W., Mi, L., Nelson, P., Hamilton, M.G., Lundy, P.M. and Sawyer, T.W. (2001). Sulphur mustard induces apoptosis in diverse cell types. *Journal of Applied Toxicology* (Accepted with revisions).

Lundy, P.M., Hamilton, M.G., Mi, L., Gong, W., Vair, C., Sawyer, T.W. and Frew, R. (2001). Stimulation of Ca2+ influx through ATP receptors on rat brain synaptosomes: Identification of functional P2X7 receptor subtypes. *British Journal of Pharmacology* (Accepted).

## CONCLUSIONS

During this past year some important new milestones have been achieved which have resulted in some exciting new initiatives. We have corrected our previous inability to demonstrate ATP toxicity and as a result have now extensively probed the biochemistry of ATP receptors and shown their relationship to cytotoxicity through a membrane bound cytolytic pore. We have also extensively characterized several factors that control receptor activity and subsequent pore dilation, including agonist and antagonist actions, ionic strength, ionic composition and pH. During these studies, it became apparent that HD was also very sensitive to some of the same factors that modulated P2X receptors. Further studies have subsequently demonstrated that the toxicity of HD is exquisitely dependent not only on the ionic environment, but also on the pH. Thus, HD toxicity is much reduced when the initial exposure is in a non-ionic or a basic environment.

We have undertaken an important new research avenue concerning the discovery and characterization of a cytolyitc pore that is linked to at least two receptors. These receptors may act independently or in concert to control the relative proportions of apoptotic versus necrotic cell death. We have initiated investigations into the role of HD on the receptors and the pore in this complex. We have also nearly finished studies into the role of calcium in HD induced cell death and the data appear to suggest that intracellular calcium is necessary for DNA fragmentation, but not for cell death. Inhibition of intracellular calcium appears to shunt the mechanism of cell death to one that is predominantly necrotic in nature, while not changing the overall toxicity. Finally, we have included in this report the final instalment of our data that identified the presence of $P2X_7$ receptors in nerve endings.

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**FIGURE LEGENDS**

Figure 1: Effect of oATP on HD-induced DNA fragmentation in CHO-K1 cells. Cell cultures were incubated with oATP for 2 hr prior to HD treatment. Five hours after HD exposure the cells were harvested. DNA was isolated and electrophoresed on 1.5 % agarose gels containing 0.5 mg/ml ethidium bromide.

Figure 2: Effect of oATP on HD-induced TUNEL reaction in CHO-K1 cells. Cell cultures were incubated with oATP for 2 hr prior to HD treatment. Five hours after HD exposure the cells were fixed and assayed for fragmentation using the TUNEL reaction.

Figure 3: Effect of oATP on HD-induced morphology in CHO-K1 cells. Cell cultures were incubated with oATP for 2 hr prior to HD treatment. Five hours after HD exposure the cells were stained with acridine orange and ethidium bromide and their morphology assessed using fluorescence microscopy.

Figure 4: Effect of oATP on HD-induced cytotoxicity in CHO-K1 cells. Cell cultures were incubated with oATP for 2 hr prior to HD treatment. 24 hours post-HD treatment cell viability was assessed using the alamarBlue cytotoxicity assay. Results are expressed as a percentage of the HD treated only $LC_{50}$ and represent the mean +/- standard deviation of three separate experiments.

Figure 5: Effect of incubation time on BzATP-induced LDH release in CHO-K1 cells. Confluent cultures of CHO-K1 cells were exposed to increasing concentrations of BzATP in defined sucrose buffer. At varying time intervals, the treatment buffer was aspirated and replaced with culture medium containing no drug. Cell death was measured 24 hours after the start of exposure using the LDH release cytotoxicity assay. One set of

samples was treated with BzATP in culture medium, and the exposure was carried out for the full 24 hours. Results are representative of several separate experiments.

Figure 6: Effect of oATP on BzATP-induced cell death in CHO-K1 cells. Confluent cultures of CHO-K1 cells were treated with oATP two hours prior to HD exposure. Cell death was measured 24 hours post-HD treatment using the alamarblue cytotoxicity assay. Data is normalized as a percent of the HD-only treated $LC_{50}$. Each bar represents the mean +/- standard deviation of three experiments.

Figure 7: Schematic diagram of the common cytolytic pore complex. This diagram shows the receptor activated cytolytic pore in CHO-K1 cells as defined by the results obtained in this report. Both receptors act as ionotropic pores, which upon stimulation, open a second site that allows entrance of larger molecular weight species. This activation is followed by cell death.

Figure 8: Ethidium bromide uptake in CHO-K1 cells following stimulation of the $P2X_7$ receptor. CHO-K1 cells were incubated in a sucrose based buffer containing 20 μM ethidium bromide. A sample of the cell suspension was examined under fluorescence microscopy (Panel A) and again 15 min after exposure to BzATP (100 μM), while the ethidium bromide uptake was being measured kinetically (Panel D). The number of fluorescent cells following BzATP treatment was markedly increased during the same time that significant fluorescence was measured in Panel D. Panel C shows the presence of CHO-K1 cells by light microscopy.

Figure 9: Effect of increasing concentrations of BzATP on $P2X_7$ receptor activation in low ionic buffer. CHO-K1 cells were exposed to increasing concentrations of BzATP (50- 200 μM) and ethidium uptake was measured for 15 min.

Figure 10: Inhibition of BzATP induced ethidium bromide uptake in CHO-K1 cells pretreated with oATP. CHO-K1 cells were incubated in sucrose based buffer containing 20 μM ethidium bromide and exposed to BzATP 100 μM for 15 min. Fluorescent cells are evident in Panel A. A second sample was pre-incubated with oATP (300 μM), a specific $P2X_7$ receptor inhibitor, 30 min prior to BzATP and shows no fluorescence either microscopically (Panel, B), or kinetically (Panel C).

Figure 11: Effect of ionic composition of buffer on BzATP induced ethidium bromide influx in CHO-K1 cells. This figure reveals the effect of ionic composition on the sensitivity of ethidium bromide uptake in CHO-K1 cells exposed to BzATP. CHOK-1 cells incubated in sucrose based buffer (SBB side A) or in ionic buffer (PSS) (Side B) were exposed to BzATP (200 μM) and ethidium bromide uptake was measured for 15 min. Cells incubated in sucrose buffer and exposed to BzATP exhibited an immediate rise in ethidium bromide uptake that continued for the period of observation, while cells exposed to BzATP in PSS failed to take up the dye.

Figure 12: Effect of external calcium concentration on ethidium bromide influx following $P2X_7$ receptor activation. CHO-K1 cells were exposed to BzATP in sucrose buffer containing ethidium bromide and no added calcium or in buffer containing 0.5 mM calcium. Ethidium bromide uptake was measured for 15 min.

Figure 13: Stimulation of ethidium bromide uptake in CHO-K1 cells exposed to maitotoxin (1 nM). CHO-K1 cells were incubated in a sucrose based buffer containing 20 μM ethidium bromide. A sample of the cells was examined using fluorescence microscopy (Panel A) and again 15 min following exposure to maitotoxin (1 nM), (panel B) while the ethidium

bromide uptake was also being measured kinetically (Panel D). Panel C shows the presence of CHOK-1 cells by light microscopy.

Figure 14: Comparison of rate of ethidium bromide uptake and concentration of maitotoxin in relation to ionic composition of buffer in CHO-K1 cells. CHO-K1 cells were incubated in sucrose buffer or in a physiological salt buffer in the presence of 1 nM maitotoxin. Side A compares ethidium bromide uptake following 10, 20, and 30 min incubation in the two buffers. Side B summarizes the concentration effect relationship at a single time of incubation (20 min). The results are from 4 separate experiments.

Figure 15: Concentration-response relationship between maitotoxin and cell death in CHO-K1 cells. CHO-K1 cells were incubated in medium containing various concentrations of maitotoxin. After 24 hrs cell toxicity was measured by alamarBlue and the $EC_{50}$ calculated.

Figure 16: Comparison of BzATP versus maitotoxin induction of ethidium bromide uptake in CHO-K1 cells. CHO-K1 cells were incubated for 1 hour with 20 μM ethidium bromide in optimal buffers for the expression of their respective effects. Responses of cells to maitotoxin (3 nM) in Panel A and BzATP (200 μM) in Panel B reflect a time-related biphasic ethidium bromide uptake.

Figure 17: Failure to inhibit ethidium bromide uptake in oATP treated cells exposed to maitotoxin in CHO-K1 cells. CHO-K1 cells were incubated in sucrose based buffer containing 20 μM ethidium bromide and exposed to maitotoxin (1 nM) for 15 min, (Panel A). A second sample was pre-incubated with oATP (300 μM) for 30 min prior to maitotoxin. Increased fluorescence measured kinetically can be seen in Panel C and Panel B after 15 min exposure to maitotoxin even in the presence of oATP.

Figure 18: Stimulation of ethidium bromide uptake in CHO-K1 cells exposed to maitotoxin (3 nM) in sucrose or PSS buffer. CHOK-1 cells were incubated in sucrose based buffer (Side A) or ionic buffer (PSS, Side B) and exposed to maitotoxin (3 nM). Ethidium bromide uptake was measured for 30 min. Maitotoxin induced an almost immediate increase in ethidium bromide uptake irrespective of the buffer. The uptake in the PSS was more rapid and reached maximal levels over the 30 min of observation.

Figure 19: Effect of external $Ca^{2+}$ concentration on maitotoxin stimulated ethidium bromide uptake in CHO-K1 cells. CHO-K1 cells were incubated in sucrose buffer with 20 μM ethidium bromide containing various external calcium concentrations. The cells were exposed to maitotoxin (1 nM) and ethidium bromide uptake was measured for 30 min. This represents a typical response in this buffer. Similar experiments have been repeated in ionic based buffer with similar results.

Figure 20: Effect of HD (400 μM) on ethidium bromide uptake in CHO-K1 cells. CHO-K1 cells were incubated in ethidium bromide (20 μM) and exposed to HD and dye uptake measured for 60 min. This experiment represents one experiment of four.

Figure 21: Fluorescence photomicrograph of CHO-K1 cells exposed to HD for 60 min. This is a fluorescence micrograph of a typical response of CHO-K1 cells following exposure to 400 μM HD for 1 hour. Four individual experiments are currently being combined and analyzed for the total fluorescence intensity.

Figure 22: Effect of HD on BzATP stimulated ethidium bromide uptake in CHO-K1 cells. CHO-K1 cells were incubated with BzATP (100μM) (A) alone for

1 hr or with HD (400 μM) for 10 min followed by and during the exposure to BzATP (B). The figure represents one of four experiments.

Figure 23: Time related influx of ethidium bromide in CHO-K1 cells exposed to BzATP alone or BzATP following preincubation with HD. Ethidium bromide uptake in CHO-K1 cells was measured in cells exposed to BzATP (100 μM) for 1 hr and repeated following a 10 min preincubation with HD 100 μM for 1 hour. These represent the mean +/- S.E.M. of 4 experiments

Figure 24: Effects of thapsigargin, BAPTA-AM and extracellular calcium on HD-induced toxicity in human skin keratinocytes. Confluent cultures of human skin keratinocytes were incubated with the test drugs for 1 hr prior to HD exposure. 48 hr post-HD exposure cell viability was assessed using the alamarBlue cytotoxicity assay. Results are expressed as a percentage of the $LC_{50}$ of HD only treated cultures and represent the mean +/- standard deviation of three experiments.

Figure 25: Effect of thapsigargin, BAPTA-AM and extracellular calcium on HD-induced DNA fragmentation (soluble DNA) in human skin keratinocytes. Proliferating cultures were labeled with $^{3}$H-thymidine for at least 24 hr before use. At confluency the cultures were incubated with the test drugs for 1 hr prior to HD exposure. 16 hr post-HD exposure DNA fragmentation was assessed as described in "Methods". Results represent the mean +/- standard deviation of three experiments.

Figure 26: Effect of thapsigargin, BAPTA-AM and extracellular calcium on HD-induced cell death as assessed morphologically. Confluent cultures of human skin keratinocytes were incubated with the test drugs for 1 hr prior to HD exposure. 16 hr post-HD exposure cell morphology was assessed

as described in "Methods". Results represent the mean +/- standard deviation of three experiments.

Figure 27: Effect of NaCl concentration on HD-induced cell death in CHO-K1 cells. Confluent cultures were rinsed twice with the test NaCl buffer prior to HD treatment in the same buffer. The cultures were changed back into culture medium 1 hr post-HD treatment and assessed for cell viability 23 hours later using the alamarBlue cytotoxicity assay. Results are normalized against the $LC_{50}$ of HD in culture medium and represent the mean +/- standard deviation of three experiments.

Figure 28: Effect of LiCl, NaCl and KCl concentration on HD-induced cell death in CHO-K1 cells. Confluent cultures were rinsed twice with the test salt buffer prior to HD treatment in the same buffer. The cultures were changed back into culture medium 1 hr post-HD treatment and assessed for cell viability 23 hours later using the alamarBlue cytotoxicity assay. Results are normalized against the $LC_{50}$ of HD in culture medium and represent the mean +/- standard deviation of three experiments.

Figure 29: Effect of RbCl, CsCl and choline Cl concentration on HD-induced cell death in CHO-K1 cells. Confluent cultures were rinsed twice with the test salt buffer prior to HD treatment in the same buffer. The cultures were changed back into culture medium 1 hr post-HD treatment and assessed for cell viability 23 hours later using the alamarBlue cytotoxicity assay. Results are normalized against the $LC_{50}$ of HD in culture medium and represent the mean +/- standard deviation of three experiments.

Figure 30: Effect of pH on HD-induced cell death in CHO-K1 cells. Confluent cultures were changed into medium of the desired pH immediately prior to HD treatment. The cultures were changed back into medium of physiological pH (7.4) 1 hr post-HD treatment and assessed for viability

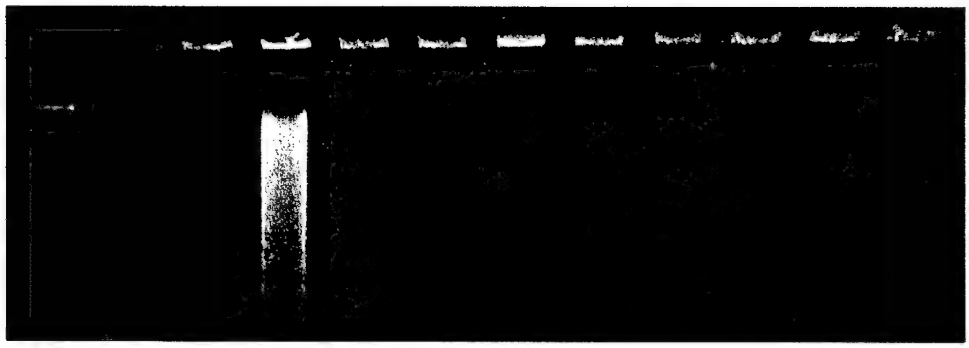
23 hr later using the alamarBlue cytotoxicity assay. Results represent the mean +/- standard deviation of three experiments.

Figure 31: Comparison of HD-induced DNA fragmentation in pH 7.5 versus pH 9.5 medium. Proliferating cultures were labeled with $^{3}$H-thymidine 24 hr prior to HD treatment. At confluency, the cultures were changed into medium of the desired pH immediately prior to HD treatment. The cultures were changed back into medium of physiological pH (7.4) 1 hr post-HD treatment and DNA fragmentation was assessed 4 hr later as described in "Methods". The data is representative of several experiments.

Figure 32: Comparison of HD-induced toxicity as measured morphologically, in pH 7.5 versus pH 9.5 medium. Confluent cultures were changed into medium of the desired pH immediately prior to HD treatment. The cultures were changed back into medium of physiological pH (7.4) 1 hr post-HD treatment and morphology was assessed 4 hr later (as described in "Methods". The data is representative of several experiments.

Figure 33: Immunoreactivity in brain slices or in purified rat brain cortical synaptosomes treated with fluorescent antibodies to $P2X_7$ receptors or the microglial marker OX-42. Panels A and B show the background fluorescence in the absence of the primary antibodies to $P2X_7$ receptors or to OX-42 in cortical sections or synaptosomes, respectively. Fluorescence is evident following treatment of cortical sections with antibodies to $P2X_7$ receptors (Panel C) or cortical synaptosomes (Panel D). Panel E reveals specific immunofluorescence in whole cortex treated with antibodies to microglial marker, OX-42. This fluorescence is absent from synaptosomal preparations (Panel F).

Fig. 1



| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HD (μM) | 0 | 200 | 400 | 600 | 0 | 200 | 400 | 600 | 0 | 200 | 400 | 600 |
| 300 μM oATP | | | | | + | + | + | + | | | | |
| 500 μM oATP | | | | | | | | | + | + | + | + |

Fig. 2

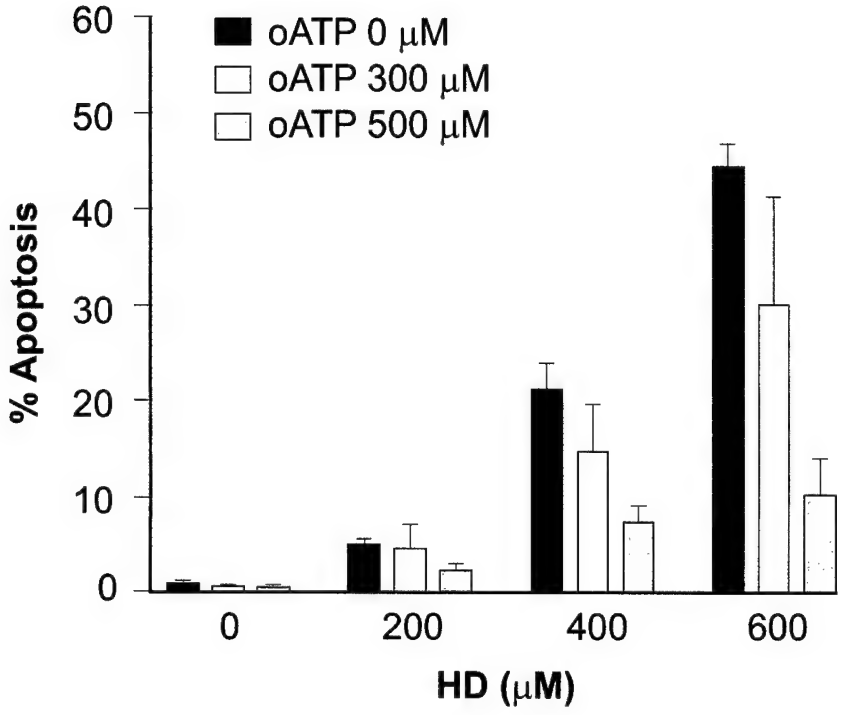

60
50
40
30
20
10
0
% Apoptosis
oATP 0 μM
oATP 300 μM
oATP 500 μM
0
200
400
600
HD (μM)

Fig. 3

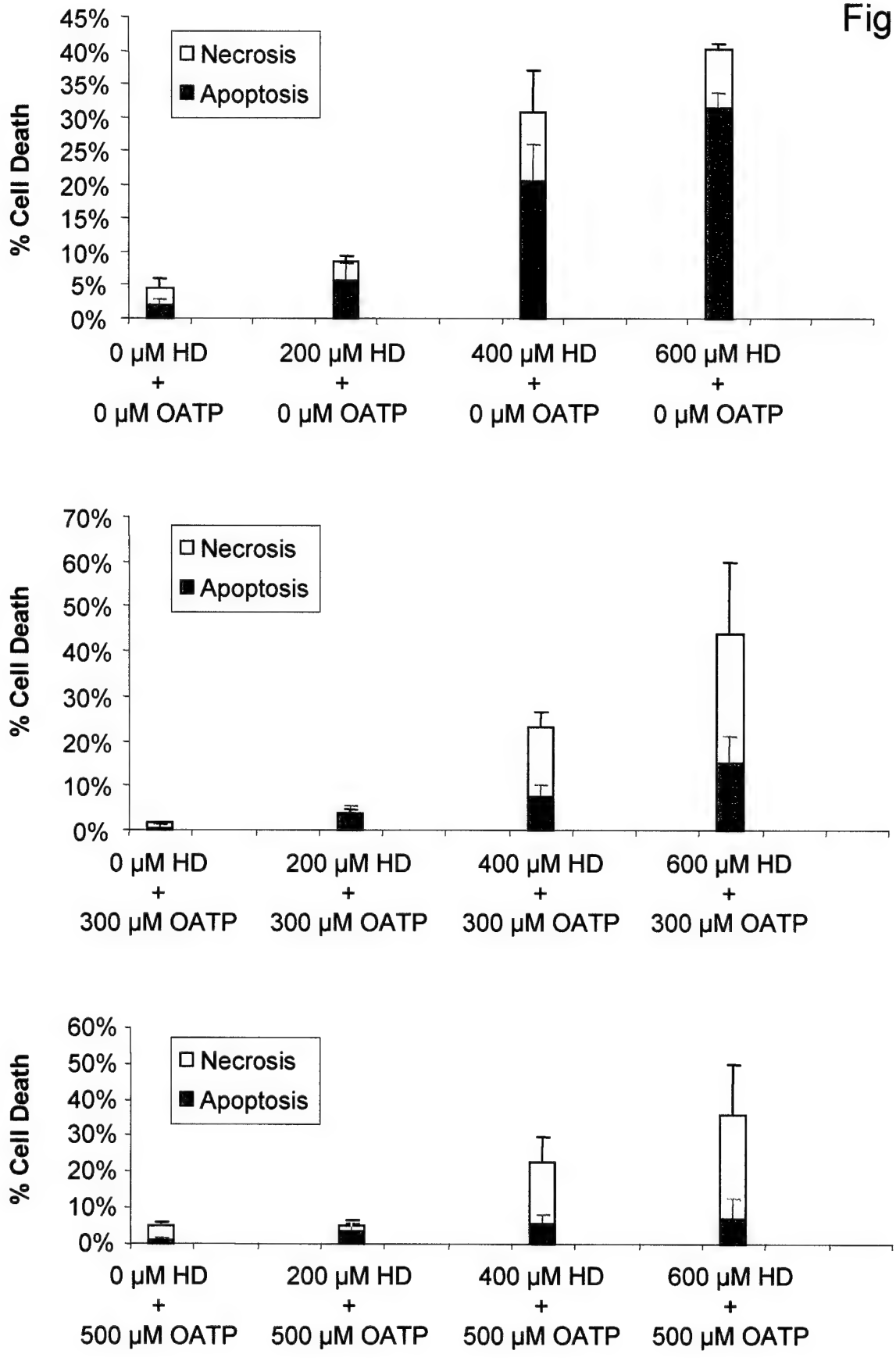

% Cell Death
Necrosis
Apoptosis
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
0 µM HD + 0 µM OATP
200 µM HD + 0 µM OATP
400 µM HD + 0 µM OATP
600 µM HD + 0 µM OATP
% Cell Death
Necrosis
Apoptosis
70%
60%
50%
40%
30%
20%
10%
0%
0 µM HD + 300 µM OATP
200 µM HD + 300 µM OATP
400 µM HD + 300 µM OATP
600 µM HD + 300 µM OATP
% Cell Death
Necrosis
Apoptosis
60%
50%
40%
30%
20%
10%
0%
0 µM HD + 500 µM OATP
200 µM HD + 500 µM OATP
400 µM HD + 500 µM OATP
600 µM HD + 500 µM OATP

Fig. 4

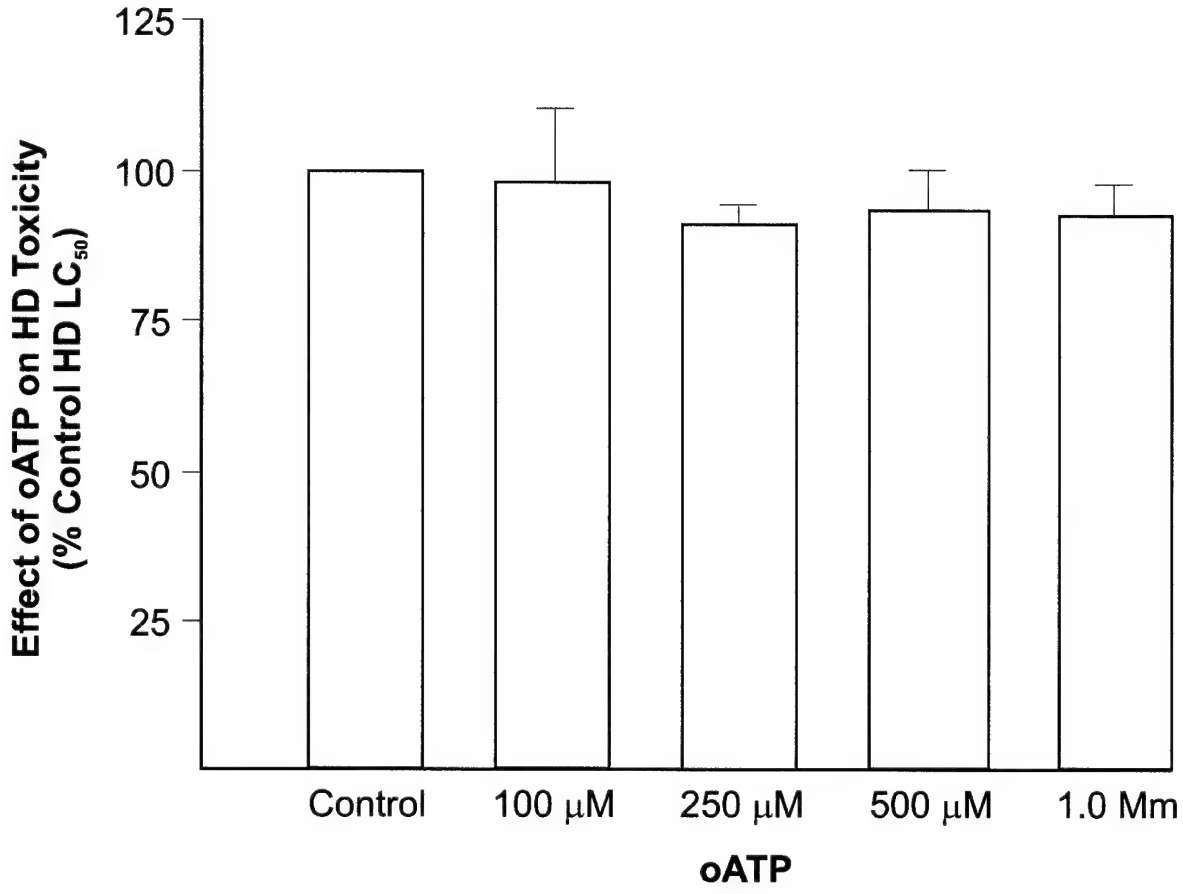

125
100
75
50
25
Effect of oATP on HD Toxicity
(% Control HD LC50)
Control
100 μM
250 μM
500 μM
1.0 Mm
oATP

Fig. 5

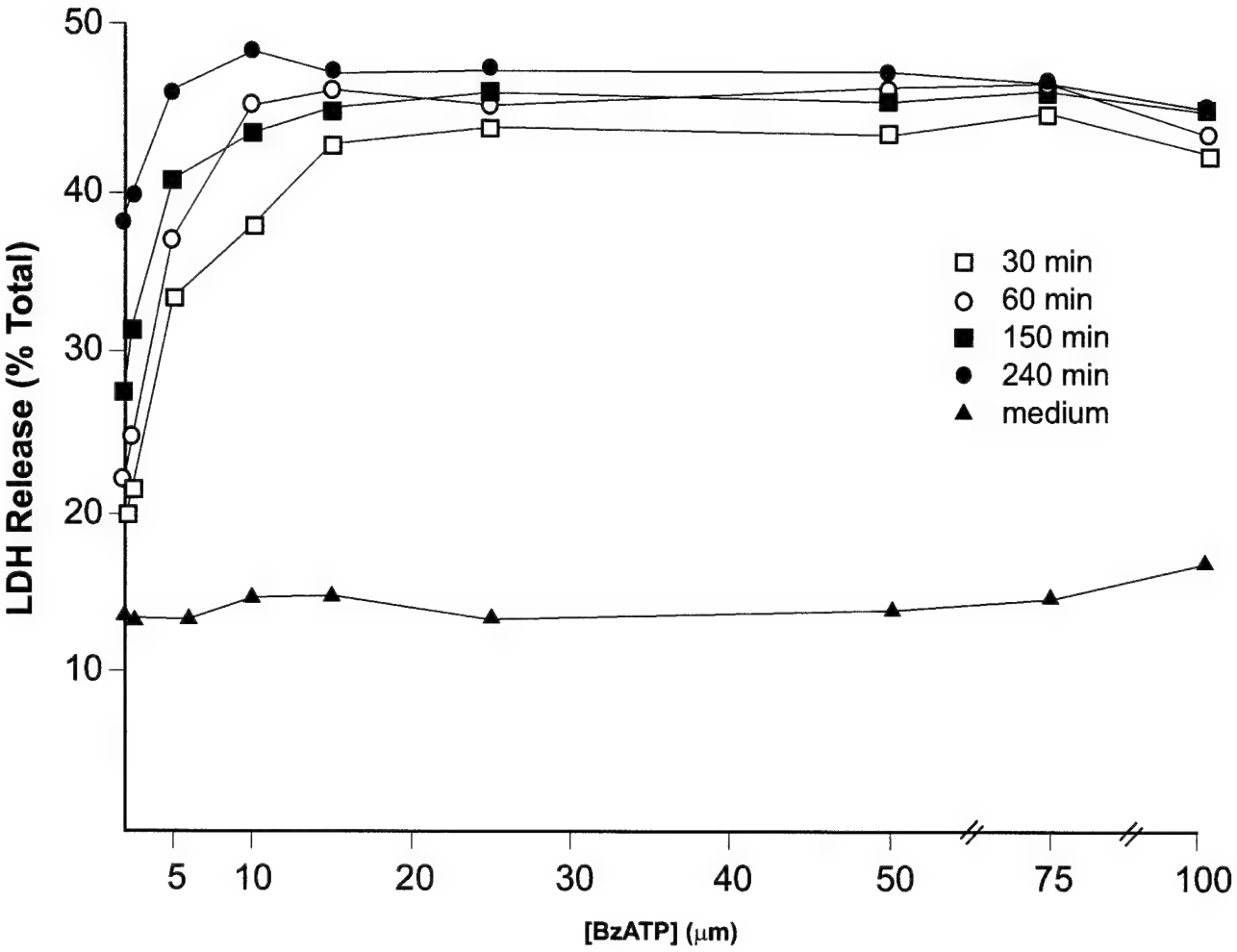

LDH Release (% Total)
50
40
30
20
10
5
10
20
30
40
50
75
100
[BzATP] (μm)
30 min
60 min
150 min
240 min
medium

Fig. 6

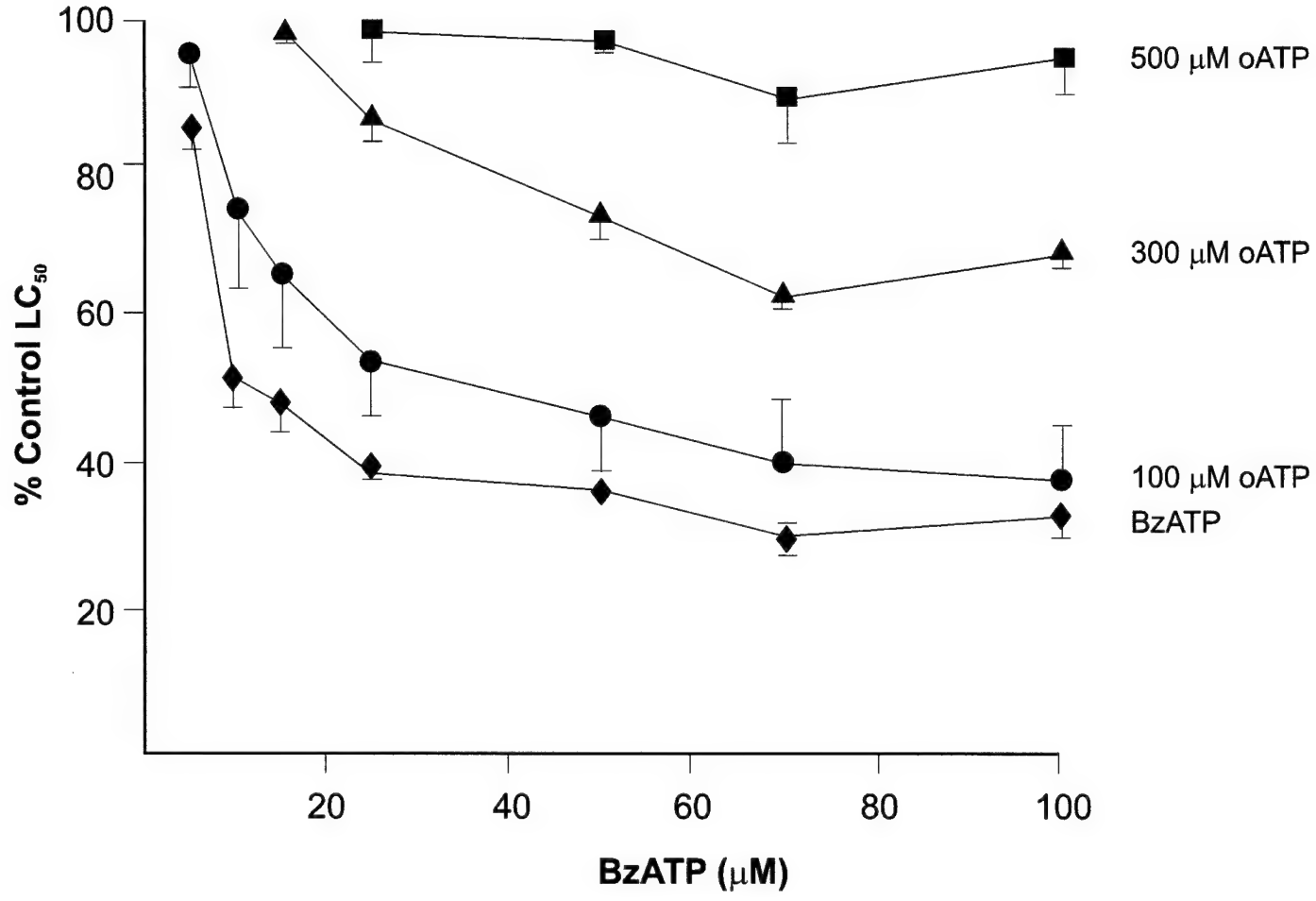

100
80
60
40
20
% Control $LC_{50}$
20
40
60
80
100
BzATP (μM)
500 μM oATP
300 μM oATP
100 μM oATP
BzATP

Fig. 7

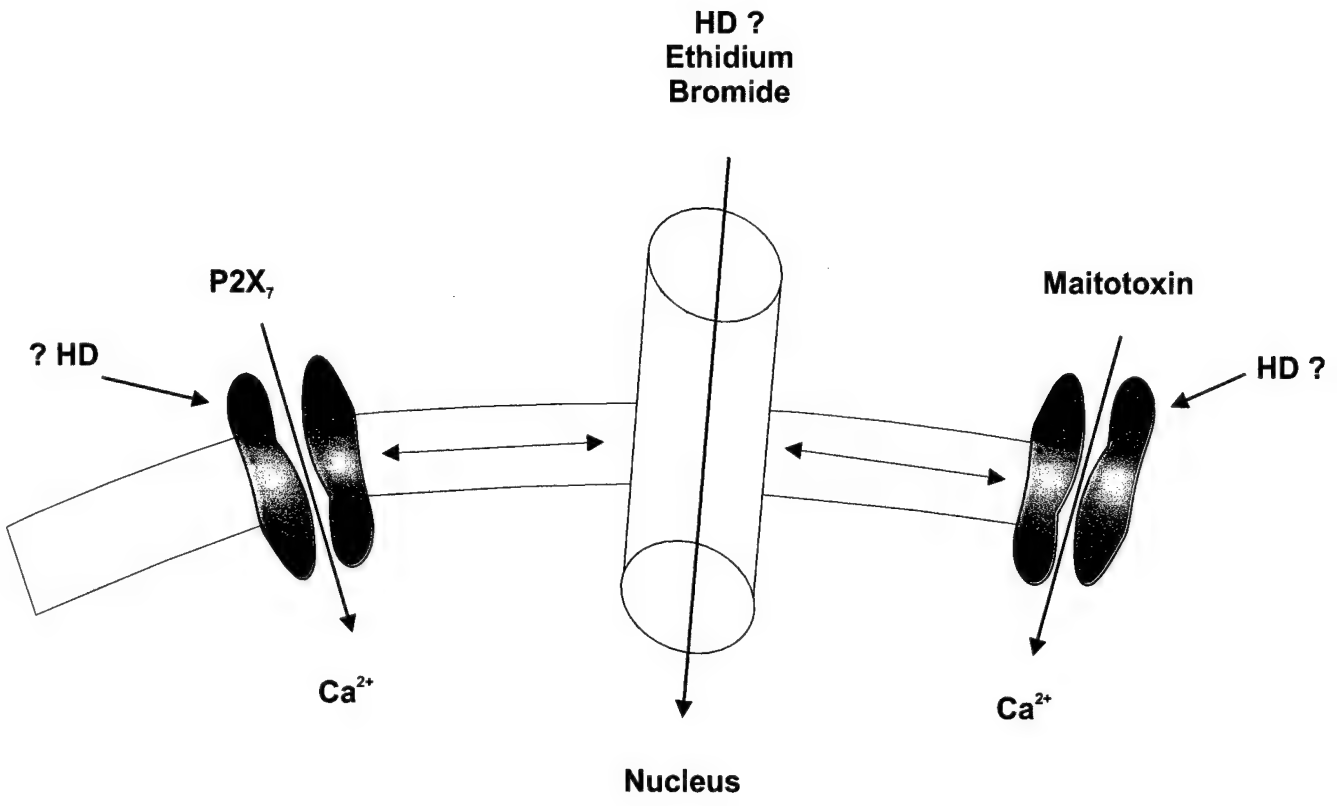


Fig. 8

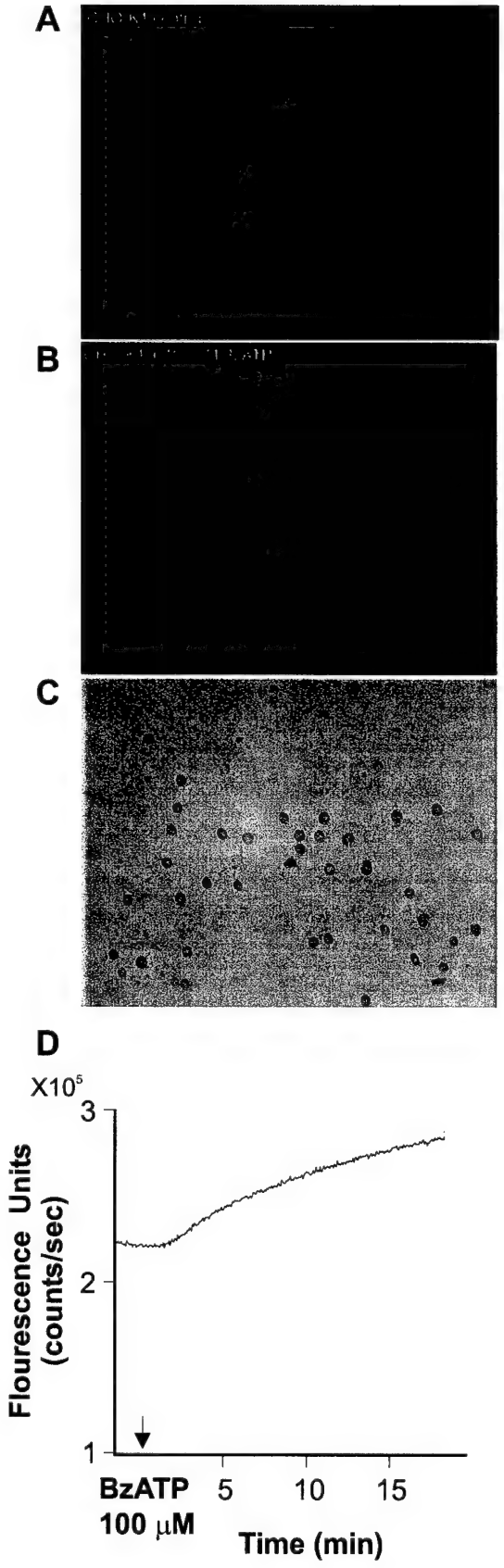

A
B
C
D
X10^5
3
2
1
Flourescence Units
(counts/sec)
BzATP
100 μM
5
10
15
Time (min)

Fig. 9

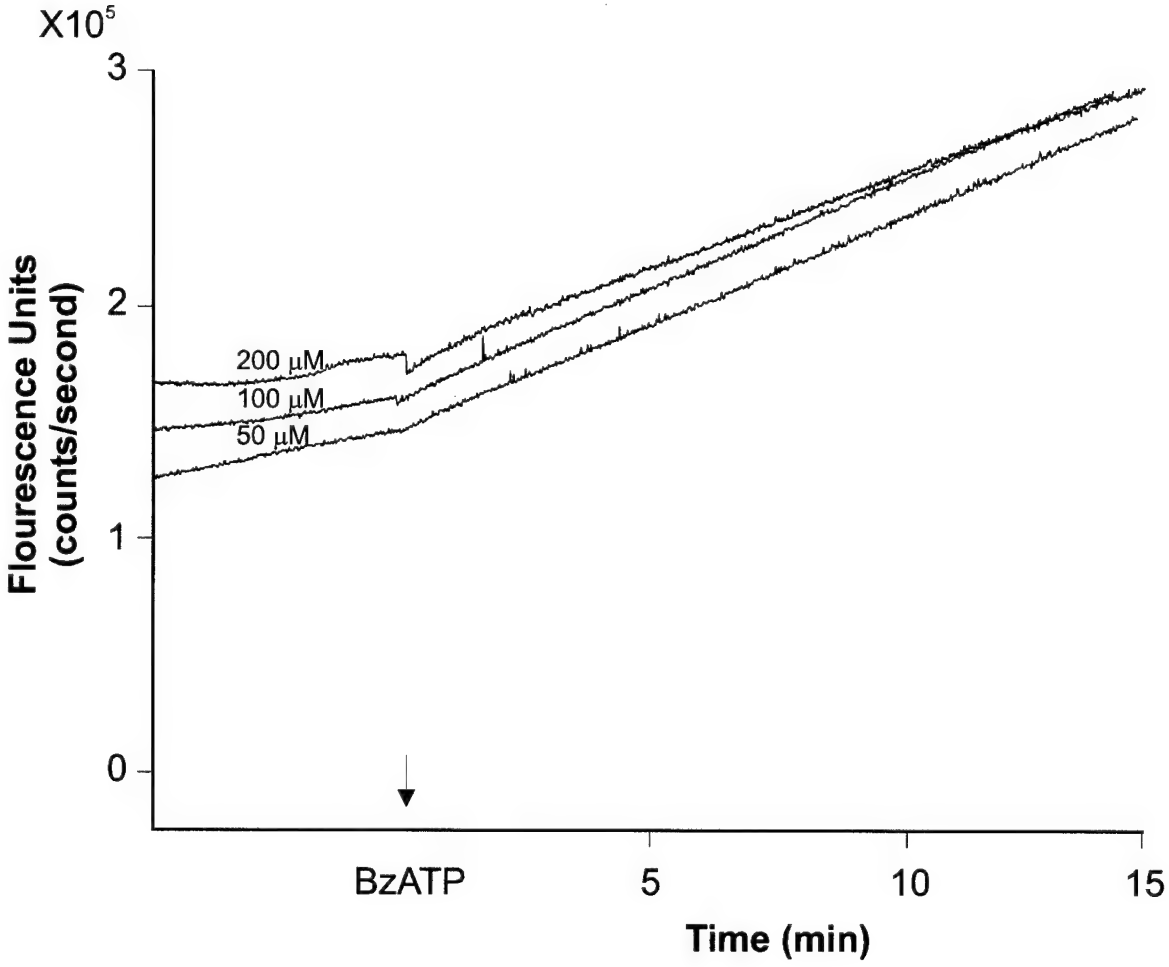

X10^5
3
2
1
0
Flourescence Units
(counts/second)
200 µM
100 µM
50 µM
BzATP
5
10
15
Time (min)

Fig. 10

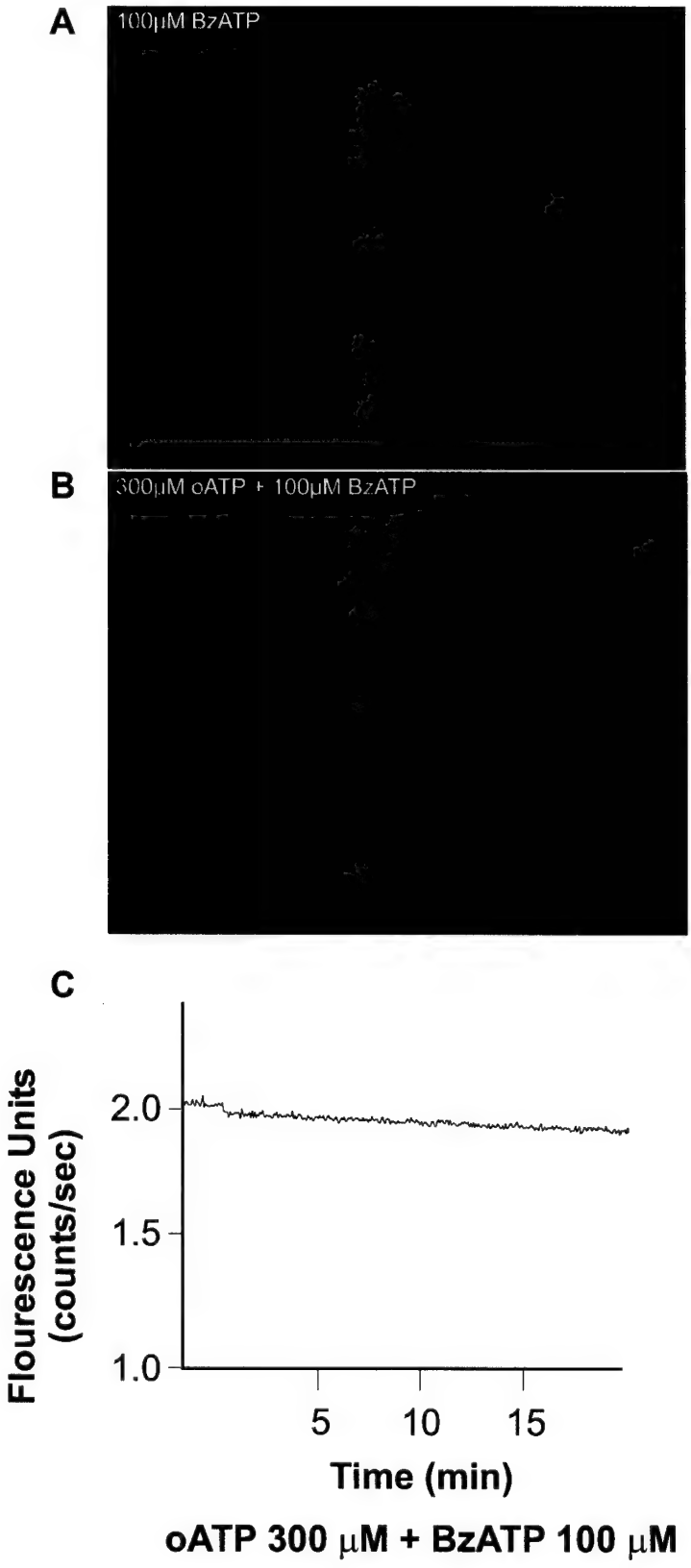
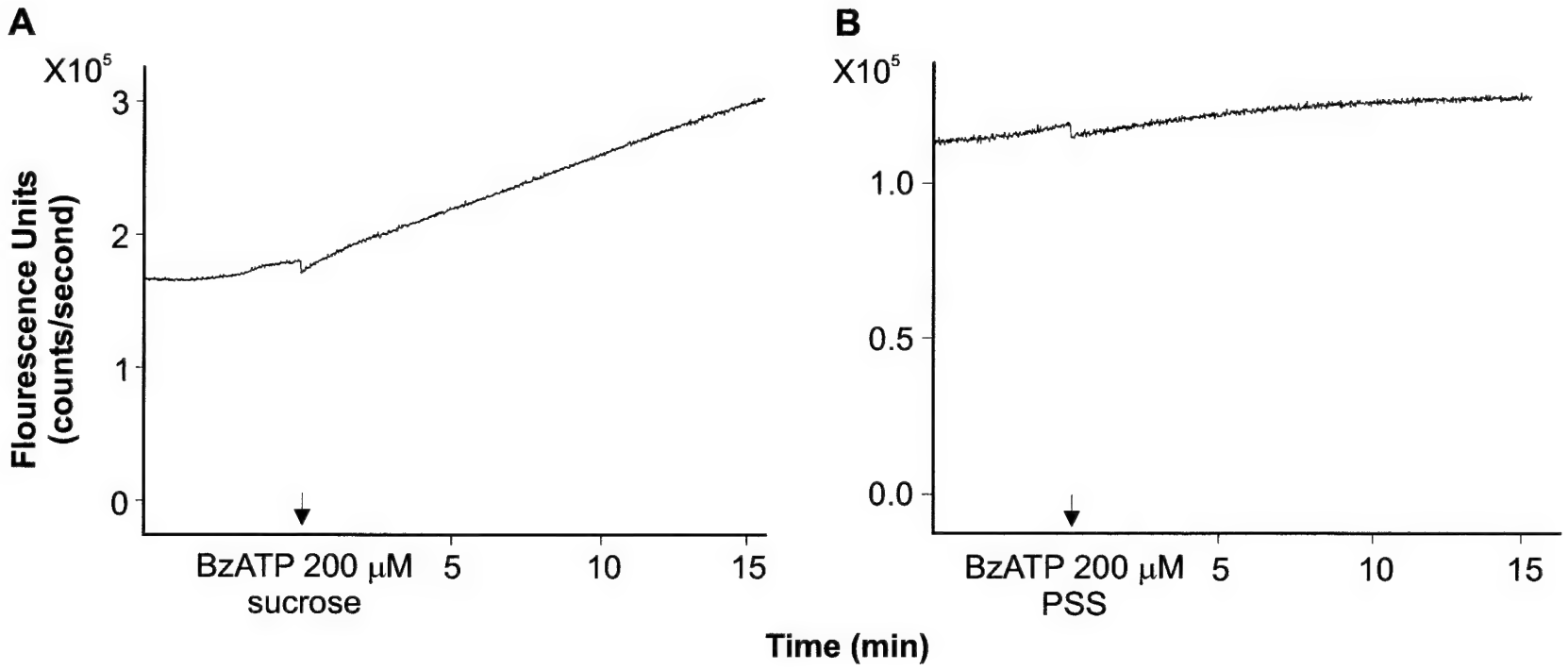

A
100μM BzATP
B
300μM oATP + 100μM BzATP
C
Flourescence Units (counts/sec)
2.0
1.5
1.0
5
10
15
Time (min)
oATP 300 μM + BzATP 100 μM

Fig. 11


A
X10^5
3
2
1
0
Flourescence Units
(counts/second)
BzATP 200 μM
sucrose
5
10
15
B
X10^5
1.0
0.5
0.0
BzATP 200 μM
PSS
5
10
15
Time (min)

99

Fig. 12

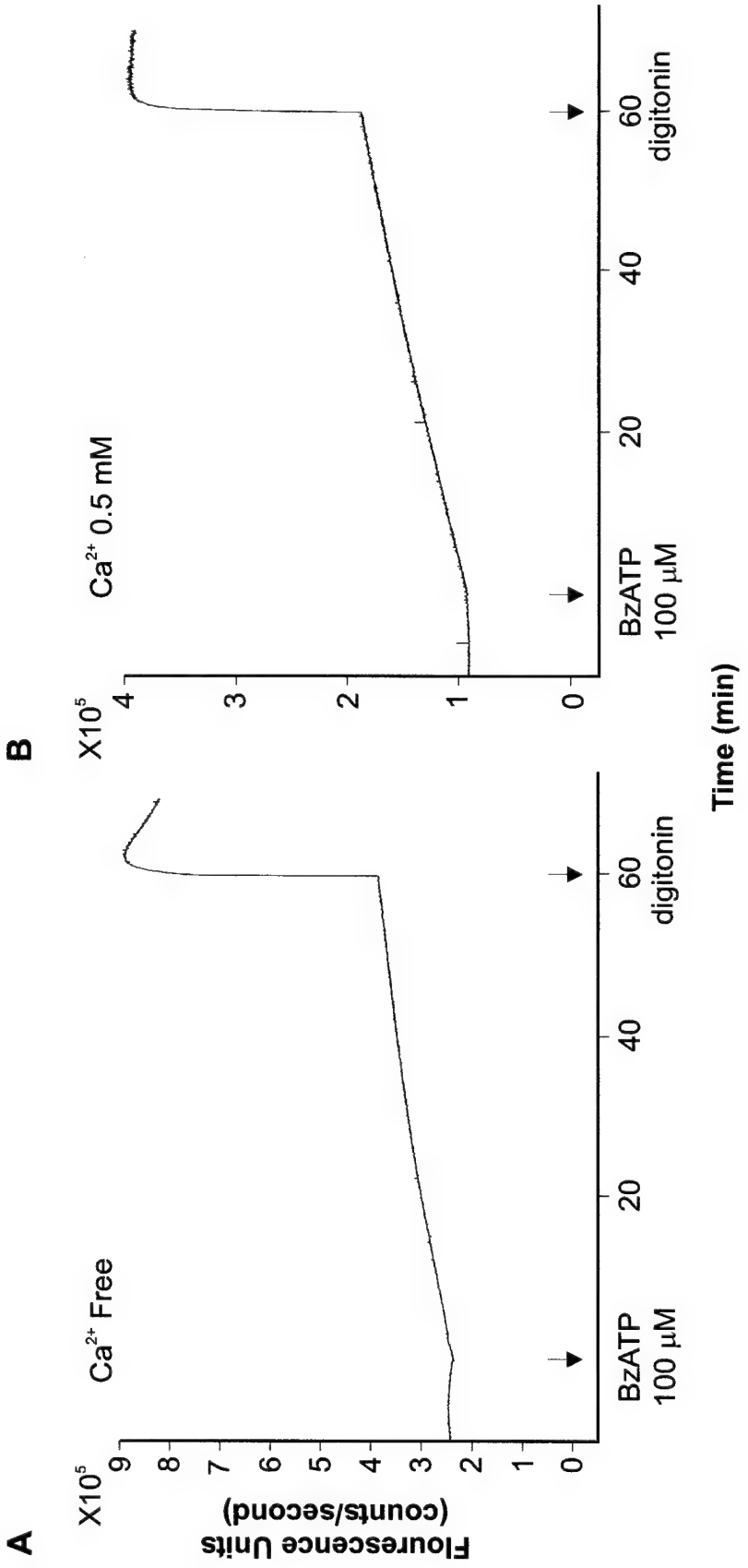

A
B
X10^5
Ca2+ Free
Ca2+ 0.5 mM
Fluorescence Units
(counts/second)
BzATP
100 µM
digitonin
Time (min)
0
1
2
3
4
5
6
7
8
9
20
40
60

Fig. 13

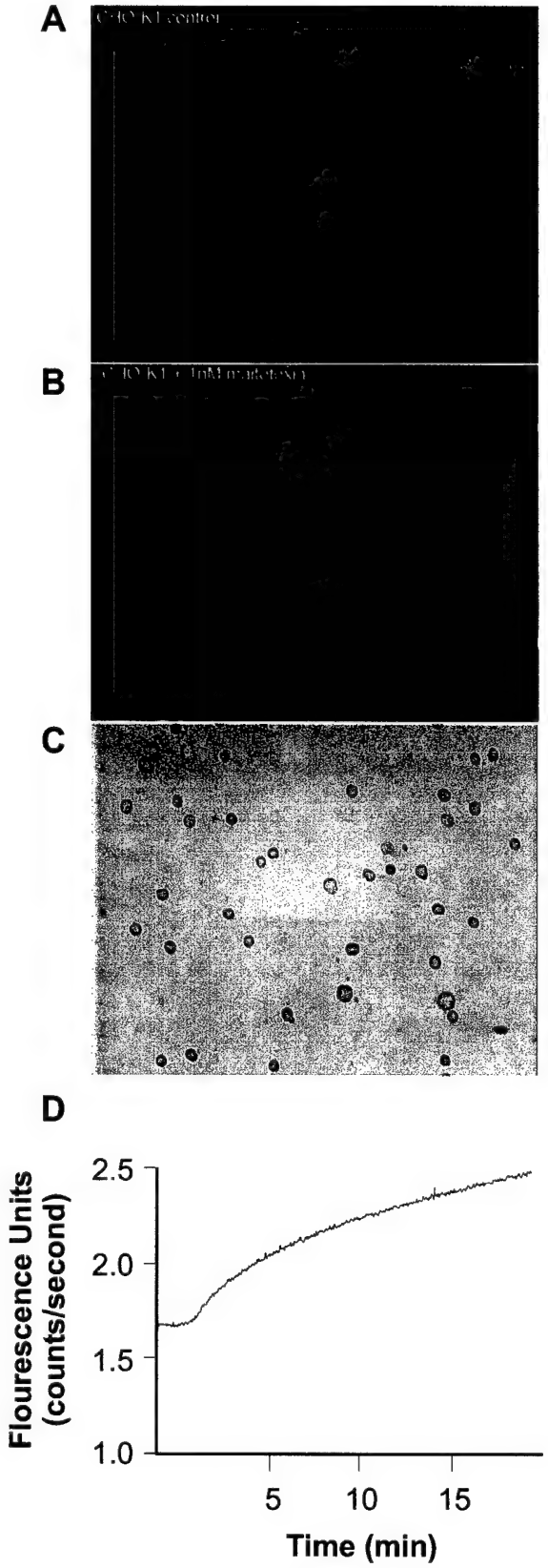

A
B
C
D
Flourescence Units
(counts/second)
2.5
2.0
1.5
1.0
5
10
15
Time (min)

Fig. 14

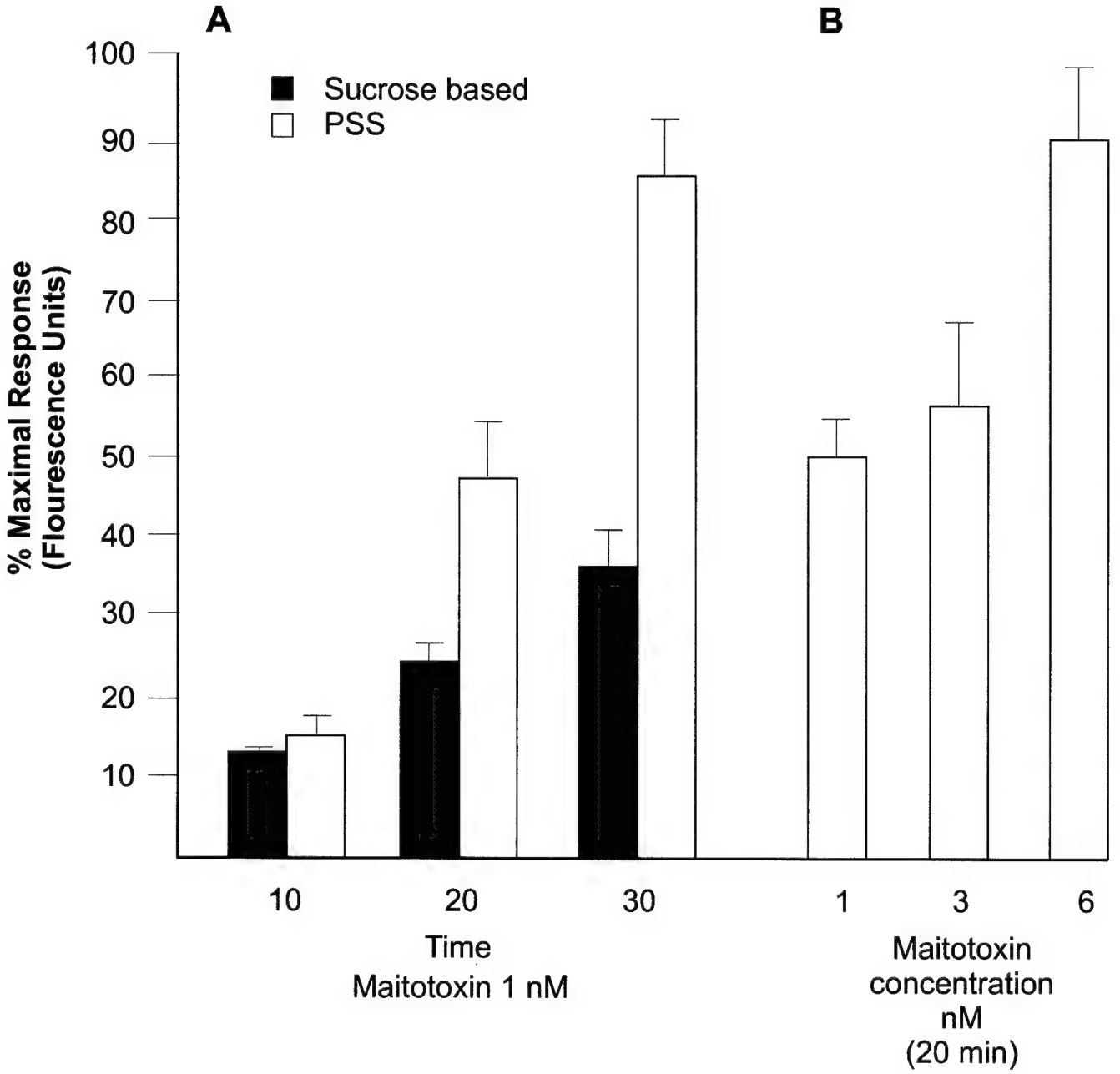

A
B
100
90
80
70
60
50
40
30
20
10
% Maximal Response
(Flourescence Units)
Sucrose based
PSS
10
20
30
1
3
6
Time
Maitotoxin 1 nM
Maitotoxin
concentration
nM
(20 min)

Fig. 15

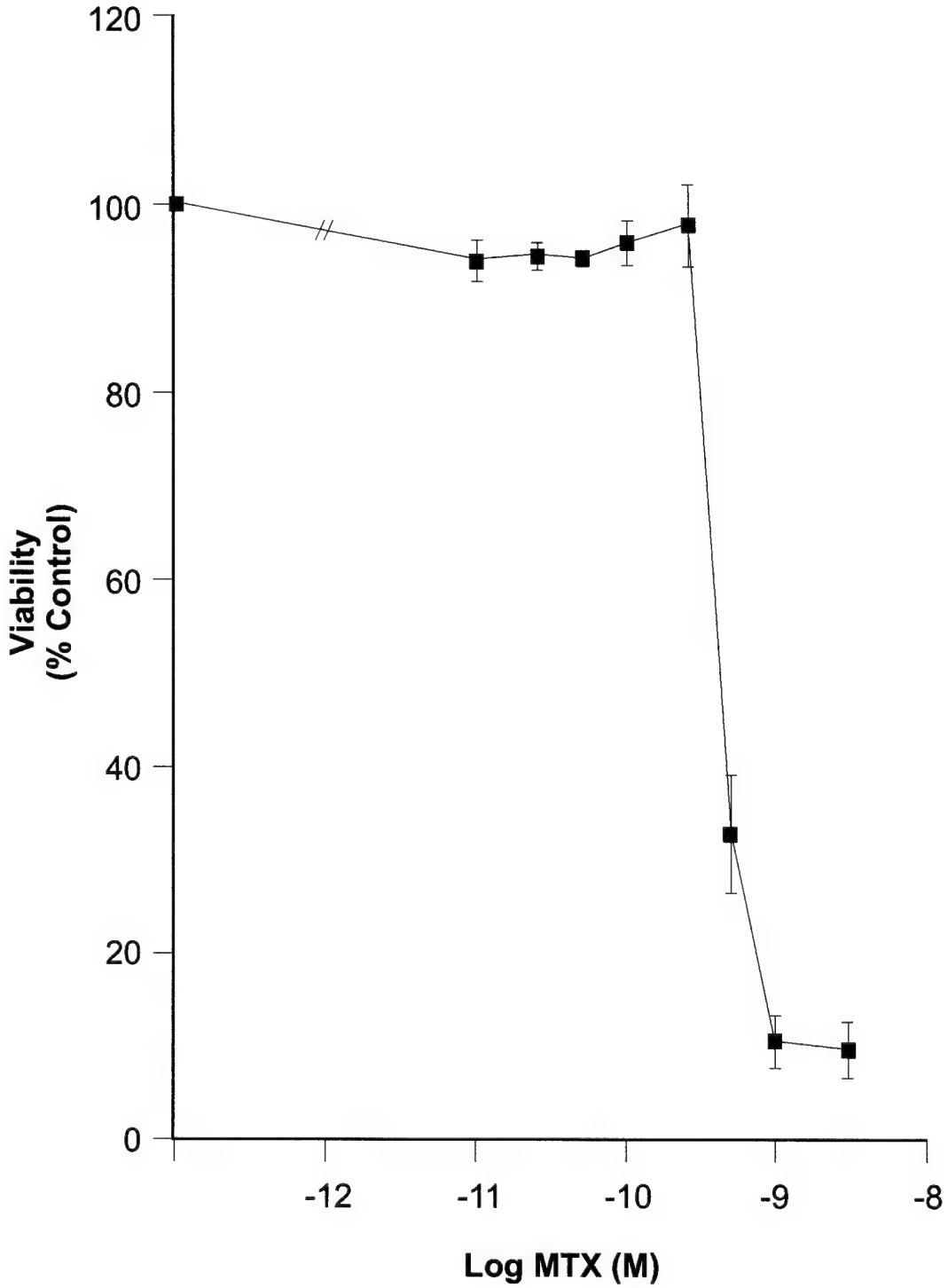


Fig. 16

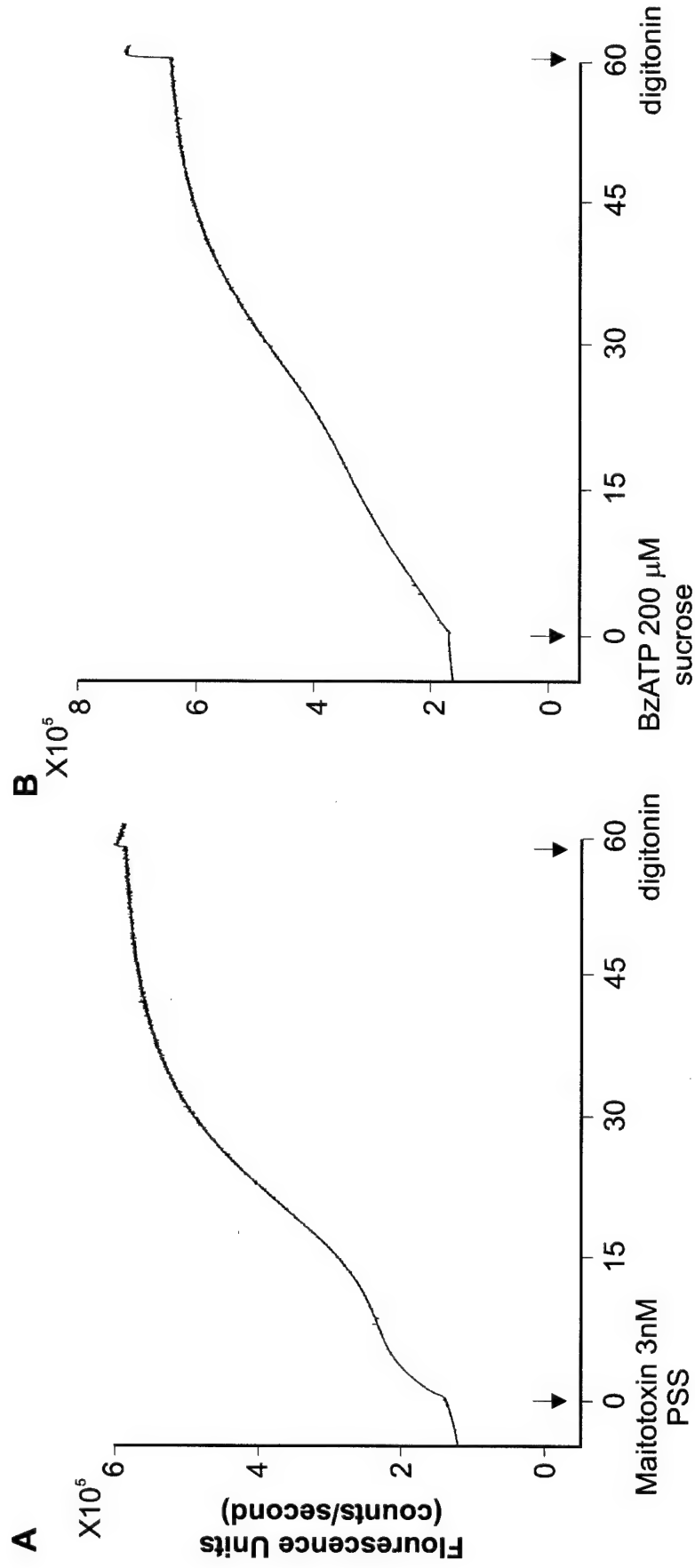

A
X10^5
6
4
2
0
Fluorescence Units
(counts/second)
0
15
30
45
60
Maitotoxin 3nM
PSS
digitonin
B
X10^5
8
6
4
2
0
0
15
30
45
60
BzATP 200 μM
sucrose
digitonin
Time (min)

Fig. 17

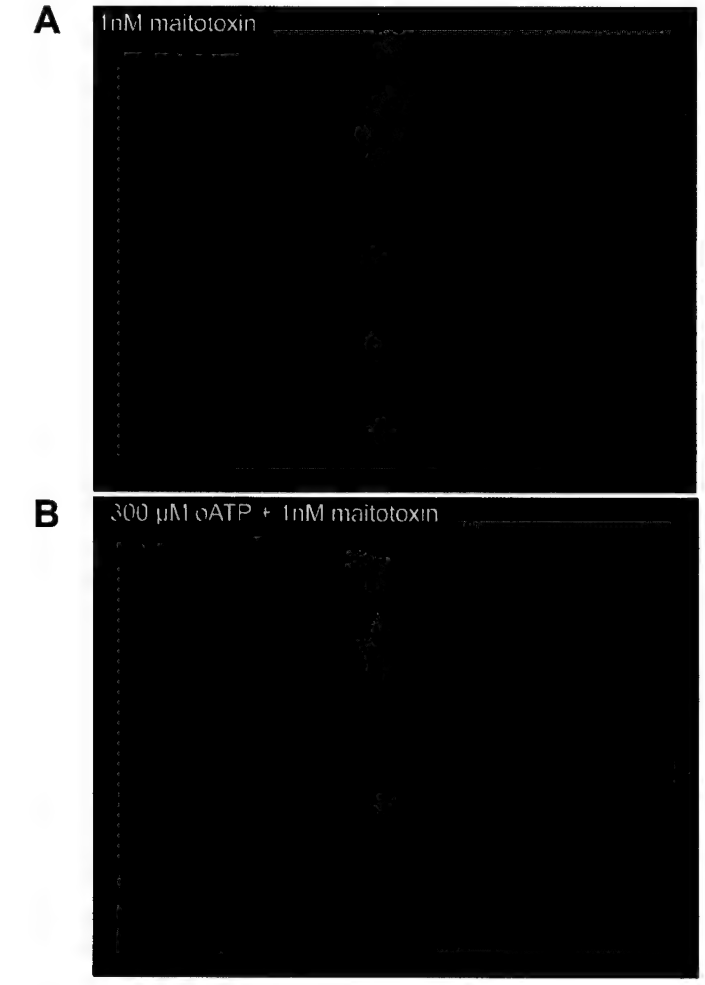

A
1nM maitotoxin
B
300 µM oATP + 1nM maitotoxin
C
Flourescence Units (counts/second)
3
2
1
5
10
15
Time (min)

Fig. 18

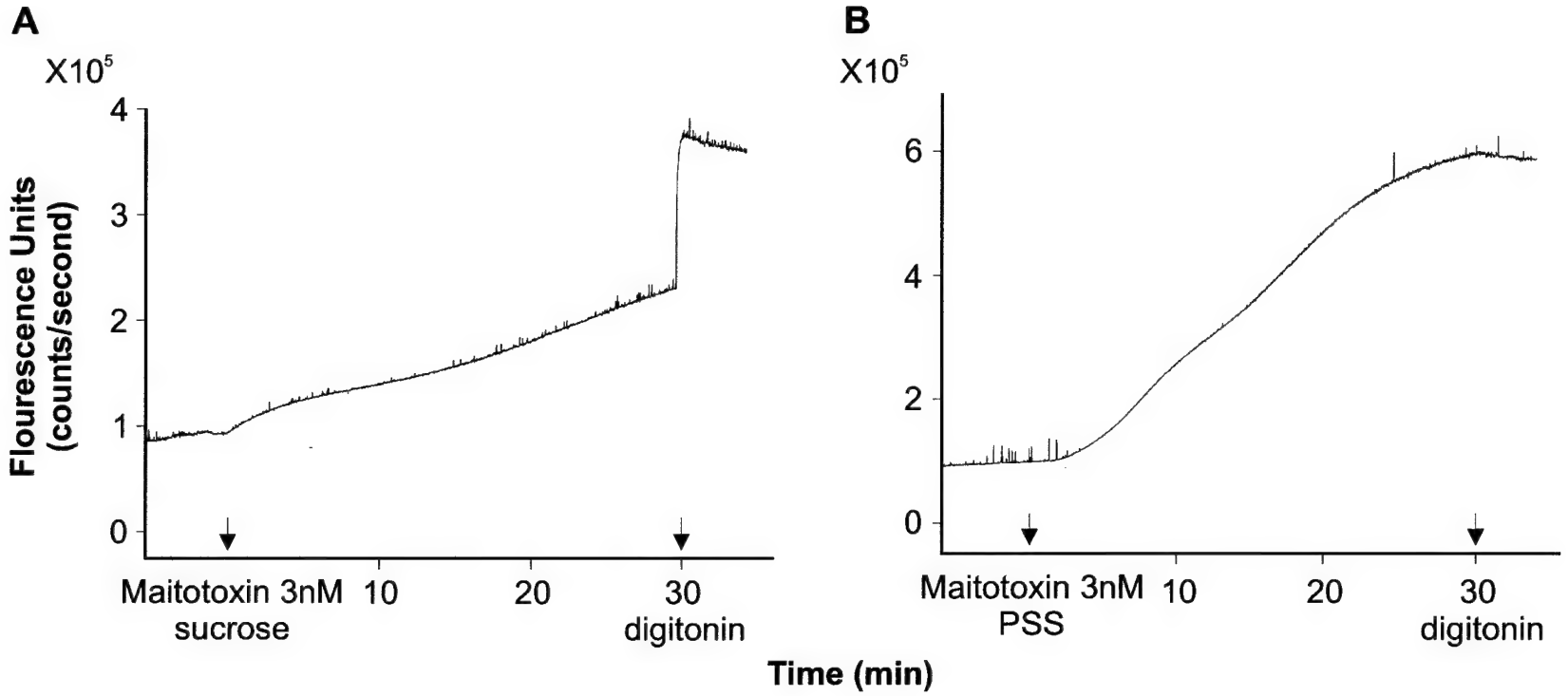

A
X10^5
4
3
2
1
0
Flourescence Units (counts/second)
Maitotoxin 3nM
sucrose
10
20
30
digitonin
B
X10^5
6
4
2
0
Maitotoxin 3nM
PSS
10
20
30
digitonin
Time (min)

Fig. 19

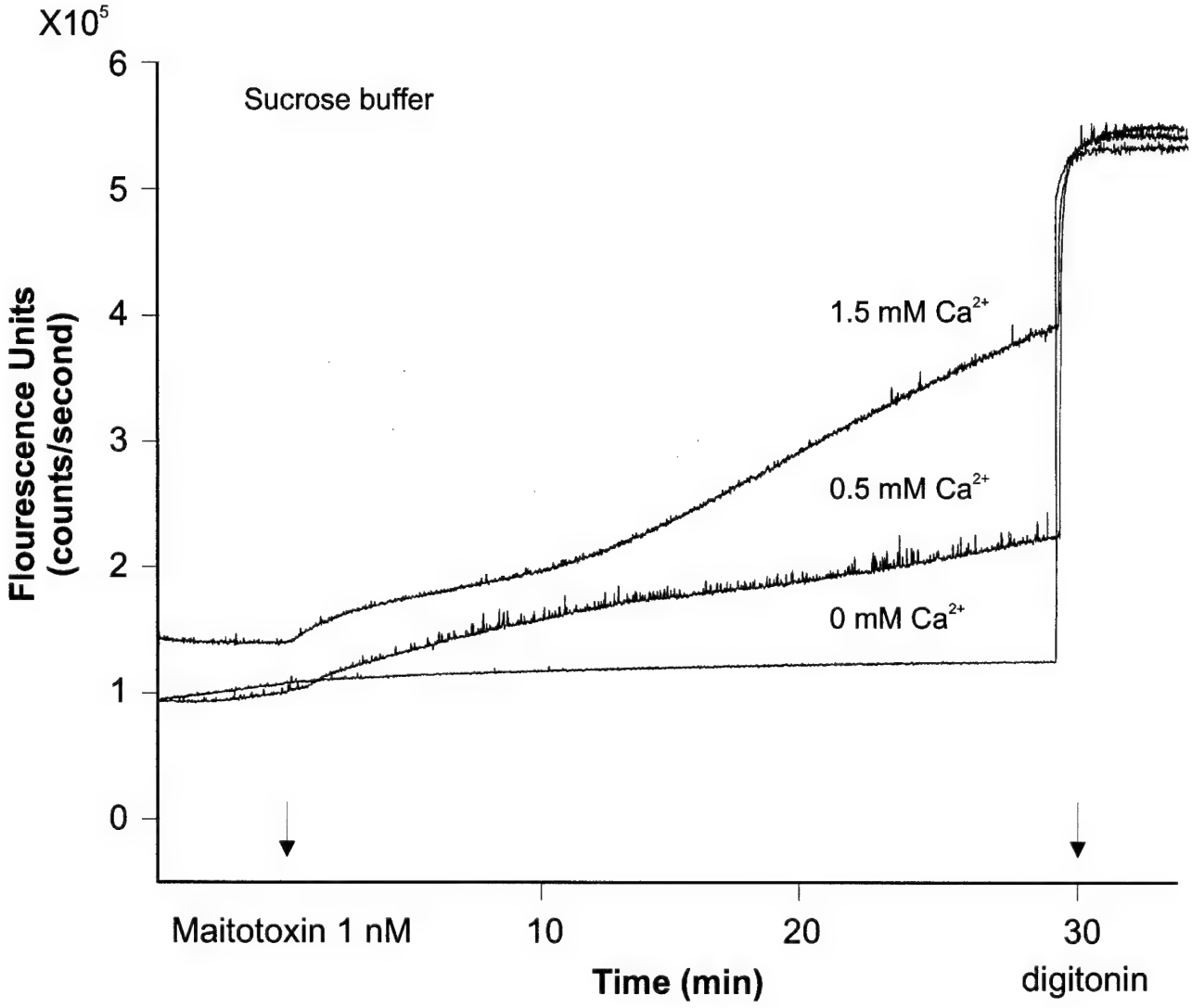

X10^5
6
5
4
3
2
1
0
Sucrose buffer
Flourescence Units (counts/second)
1.5 mM Ca2+
0.5 mM Ca2+
0 mM Ca2+
Maitotoxin 1 nM
10
20
30
digitonin
Time (min)

Fig. 20

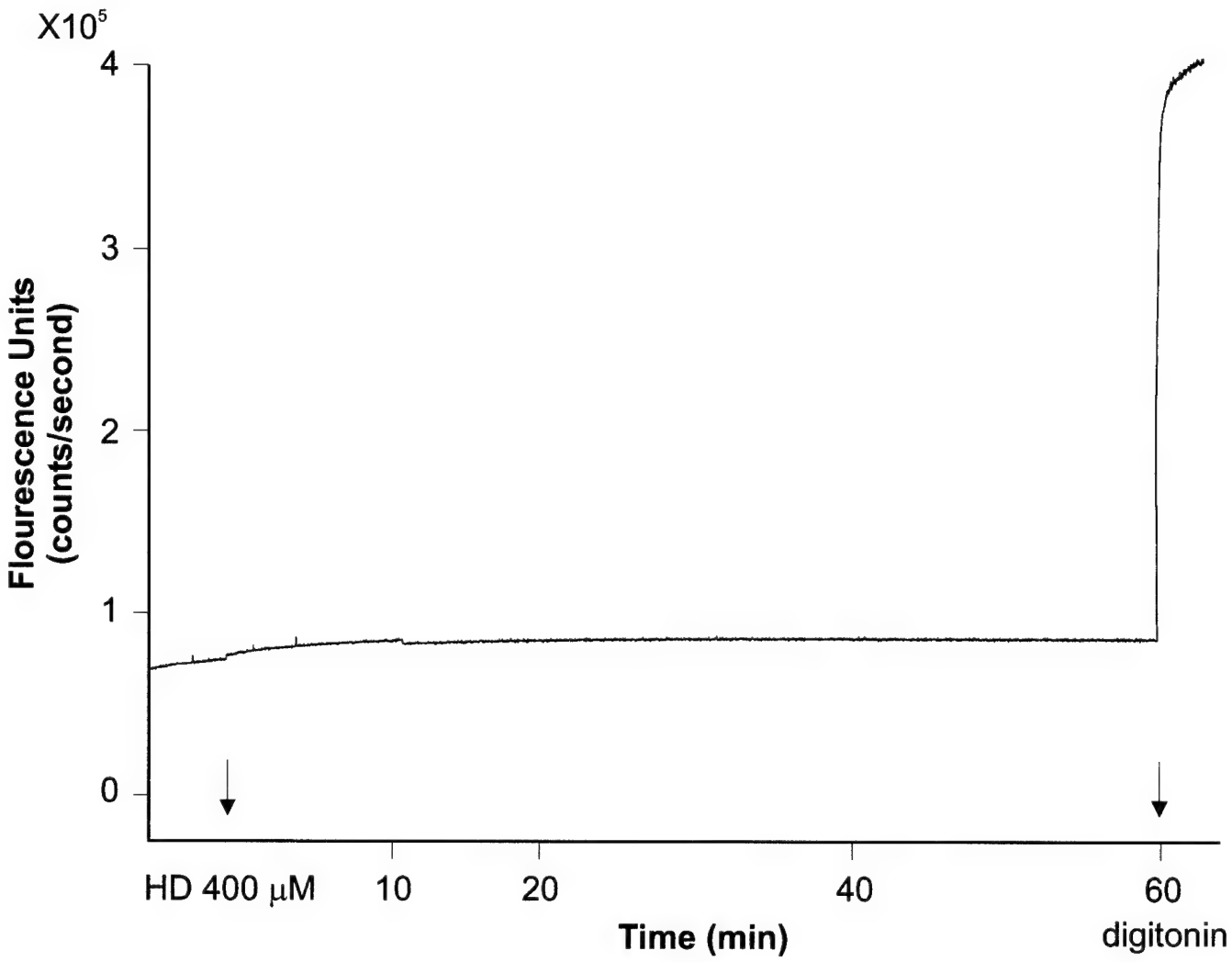

X10^5
4
3
2
1
0
Flourescence Units
(counts/second)
HD 400 μM
10
20
40
60
digitonin
Time (min)

Fig. 21

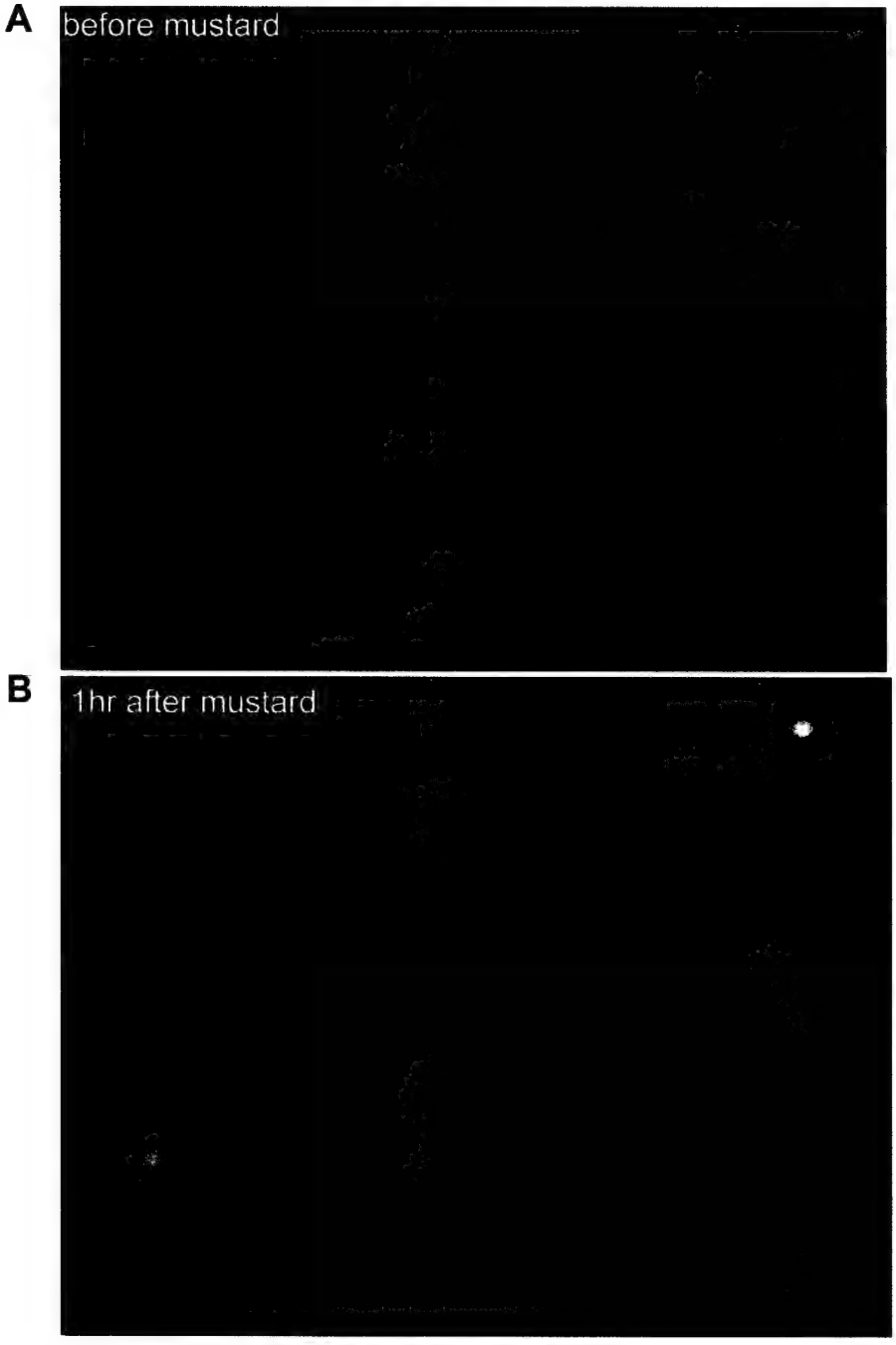


Fig. 22

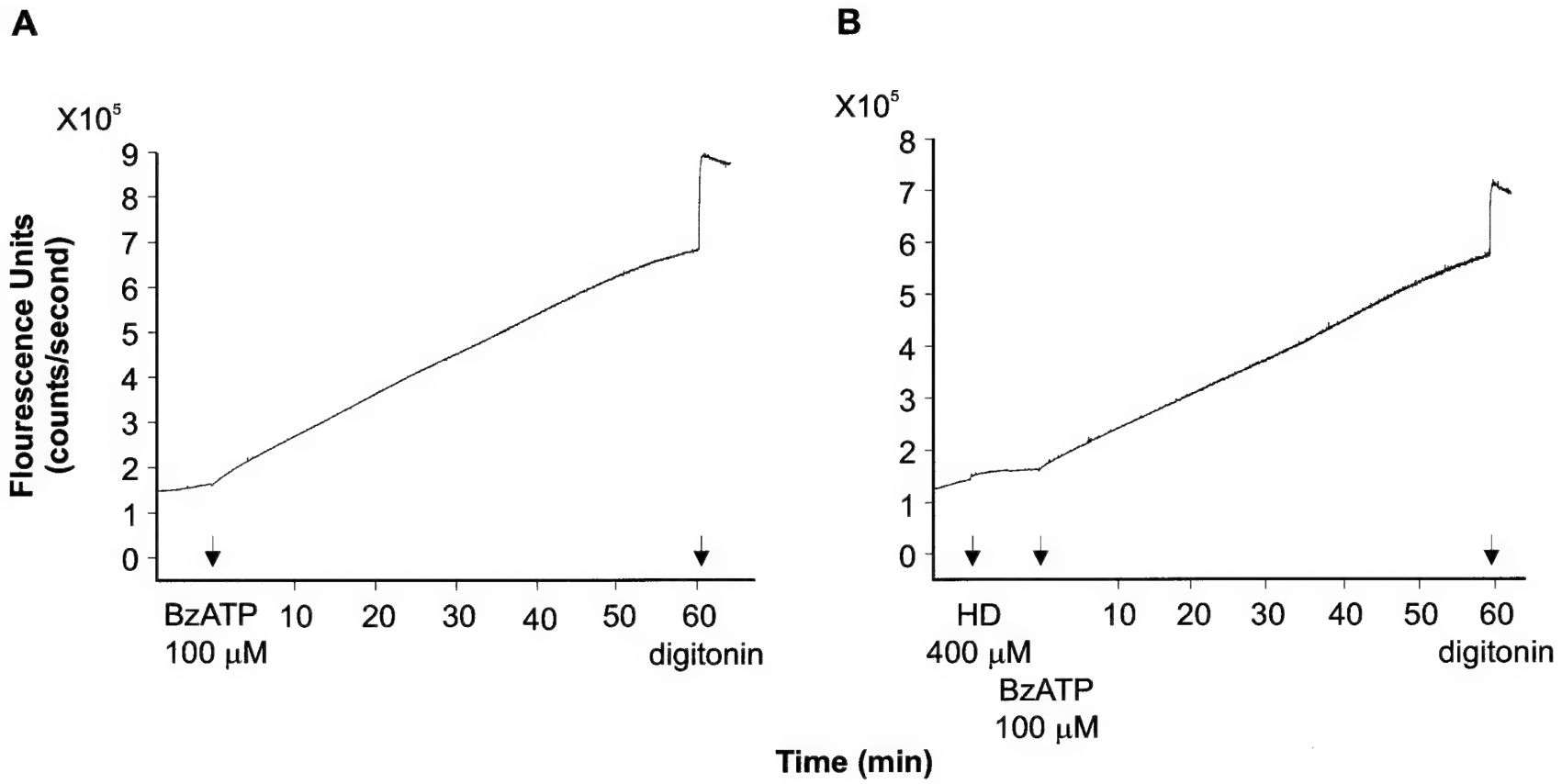

A
X10^5
9
8
7
6
5
4
3
2
1
0
Flourescence Units (counts/second)
BzATP 100 µM
10
20
30
40
50
60
digitonin
B
X10^5
8
7
6
5
4
3
2
1
0
HD 400 µM
BzATP 100 µM
10
20
30
40
50
60
digitonin
Time (min)

Fig. 23

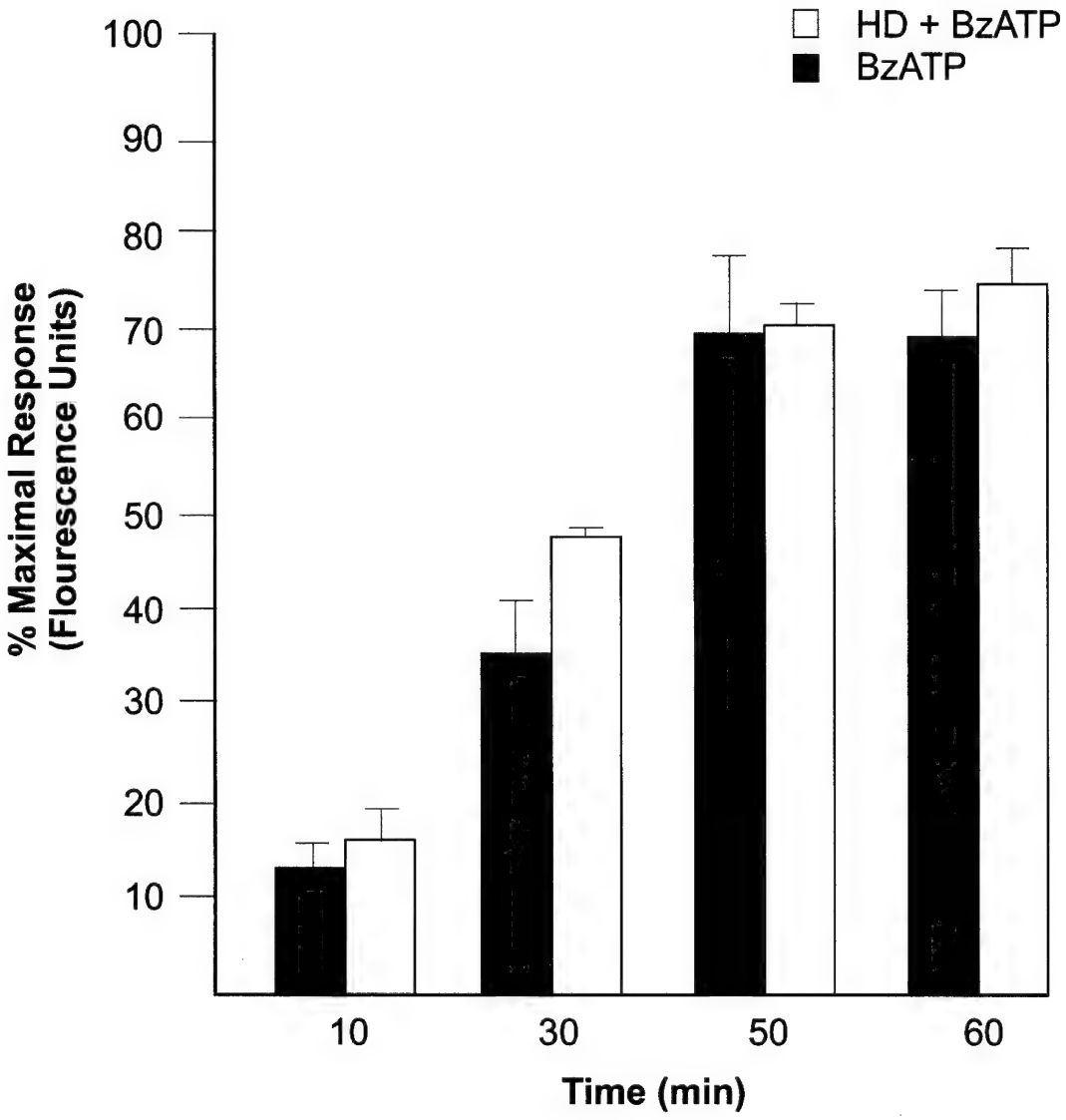
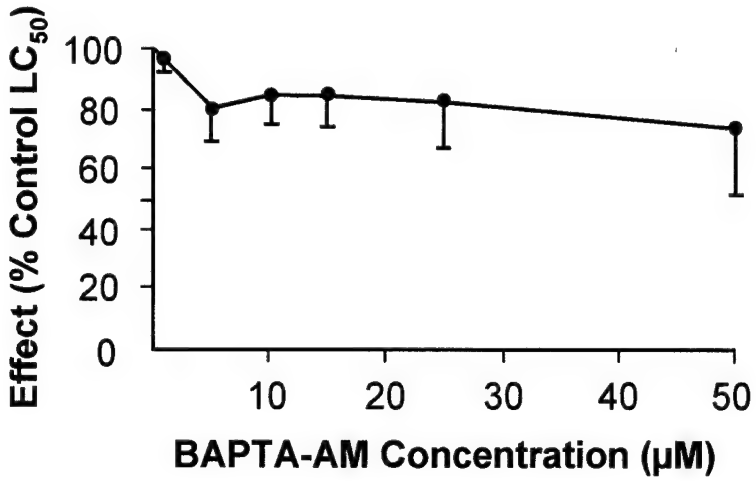
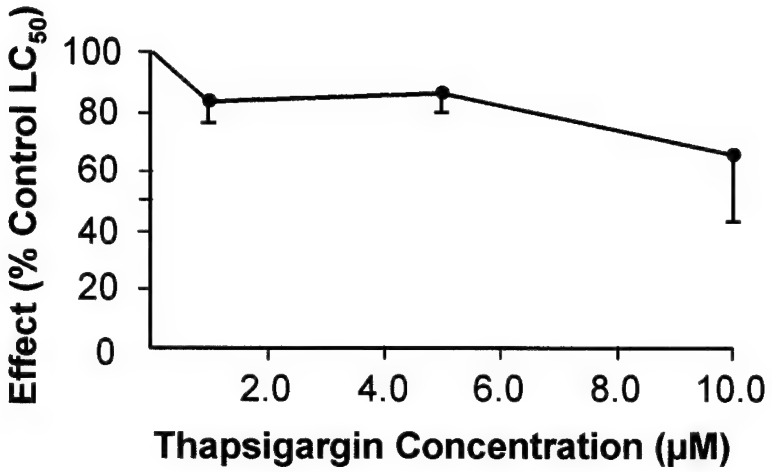

HD + BzATP
BzATP
100
90
80
70
60
50
40
30
20
10
% Maximal Response
(Flourescence Units)
10
30
50
60
Time (min)

Fig. 24

A


Effect (% Control $LC_{50}$)
100
80
60
40
20
0
10
20
30
40
50
BAPTA-AM Concentration (μM)

B


Effect (% Control $LC_{50}$)
100
80
60
40
20
0
2.0
4.0
6.0
8.0
10.0
Thapsigargin Concentration (μM)

C

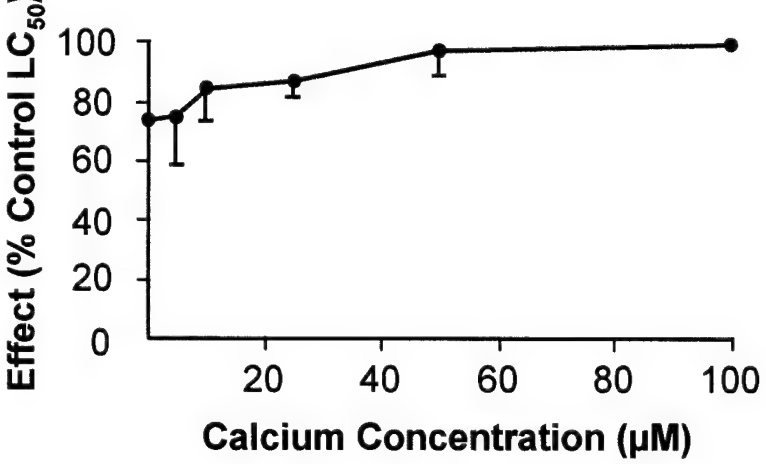

Effect (% Control $LC_{50}$)
100
80
60
40
20
0
20
40
60
80
100
Calcium Concentration (μM)

Fig. 25

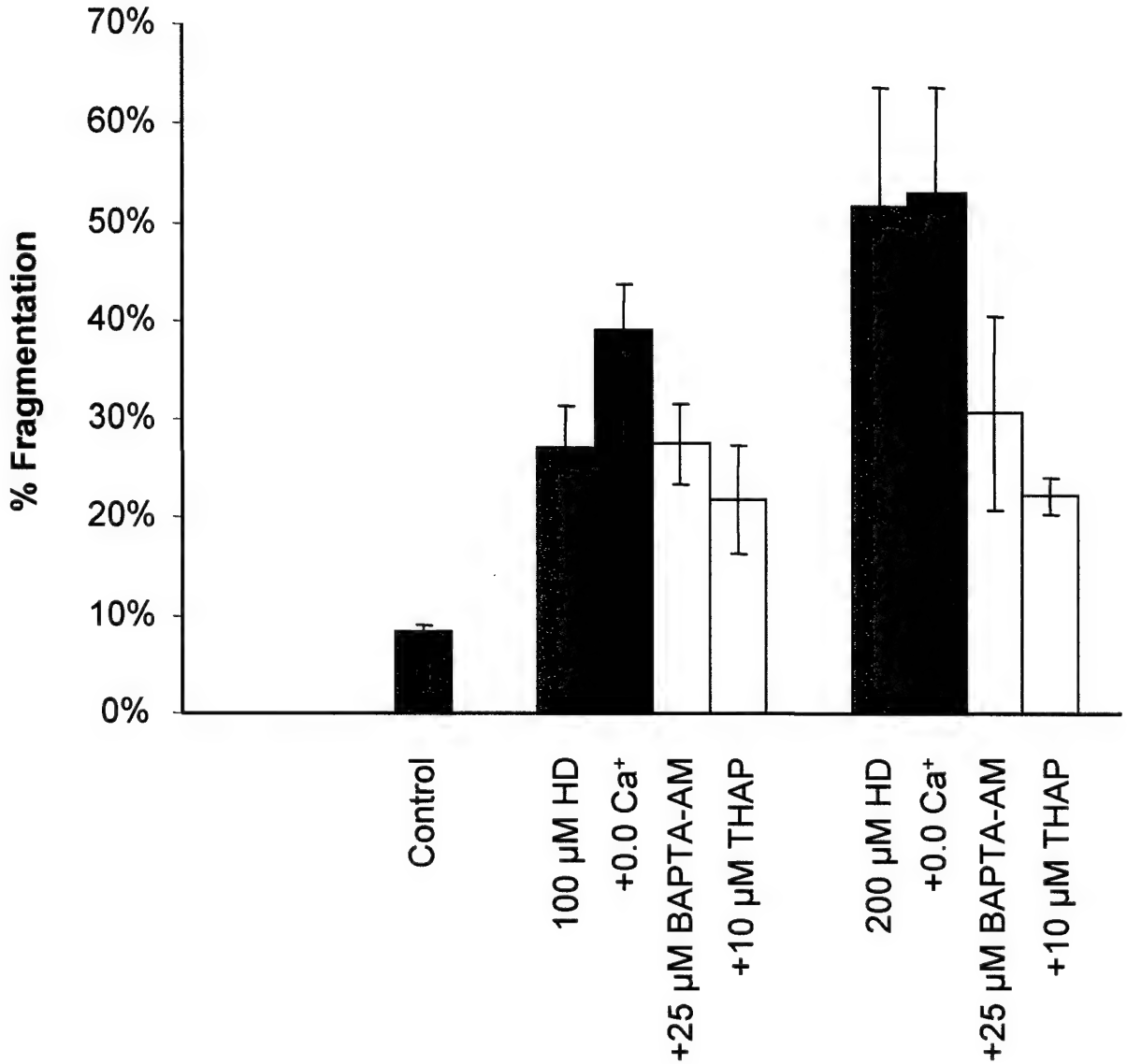

% Fragmentation
70%
60%
50%
40%
30%
20%
10%
0%
Control
100 µM HD
+0.0 Ca+
+25 µM BAPTA-AM
+10 µM THAP
200 µM HD
+0.0 Ca+
+25 µM BAPTA-AM
+10 µM THAP

Fig. 26

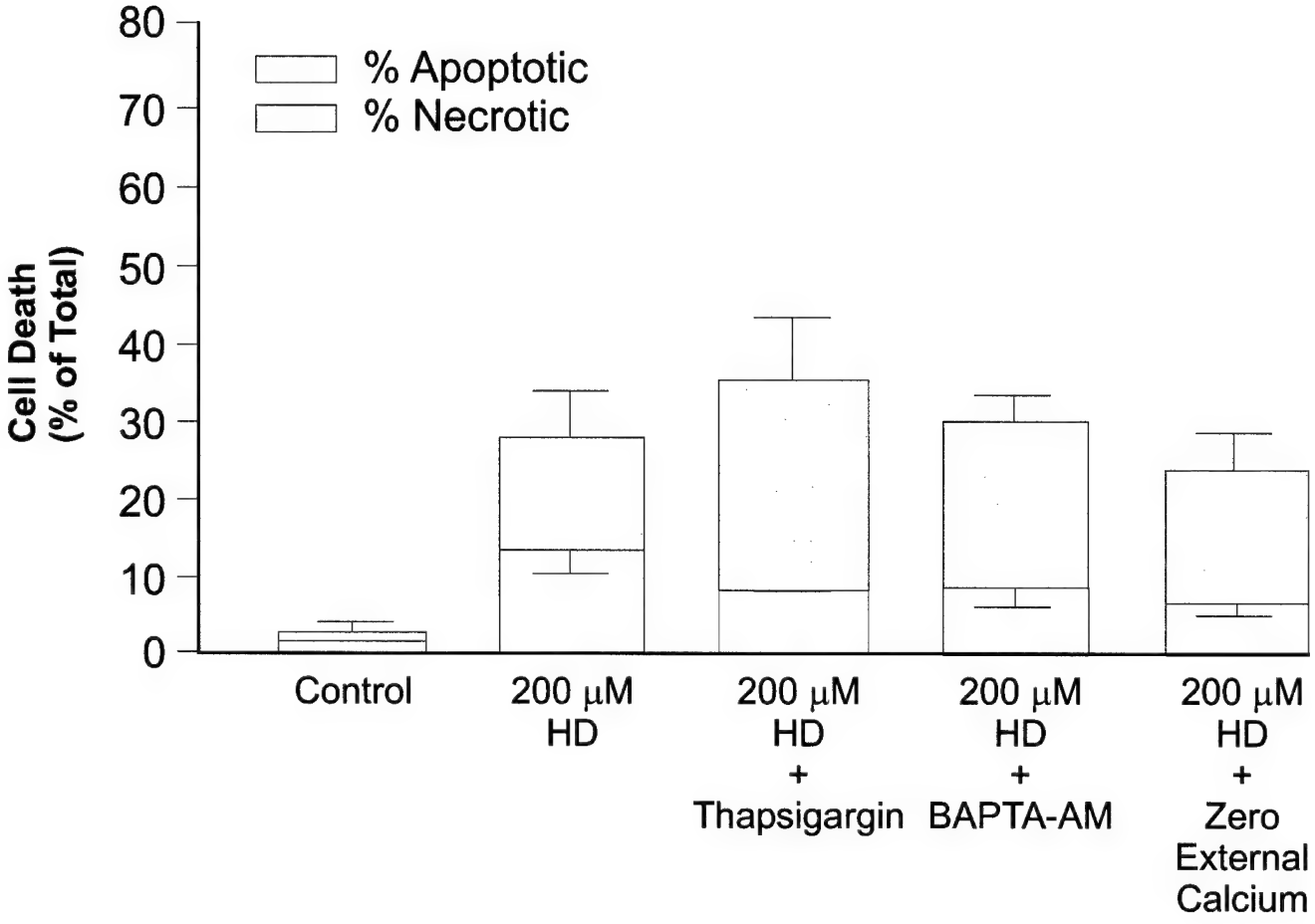

80
70
60
50
40
30
20
10
0
Cell Death
(% of Total)
% Apoptotic
% Necrotic
Control
200 μM HD
200 μM HD + Thapsigargin
200 μM HD + BAPTA-AM
200 μM HD + Zero External Calcium

Fig. 27

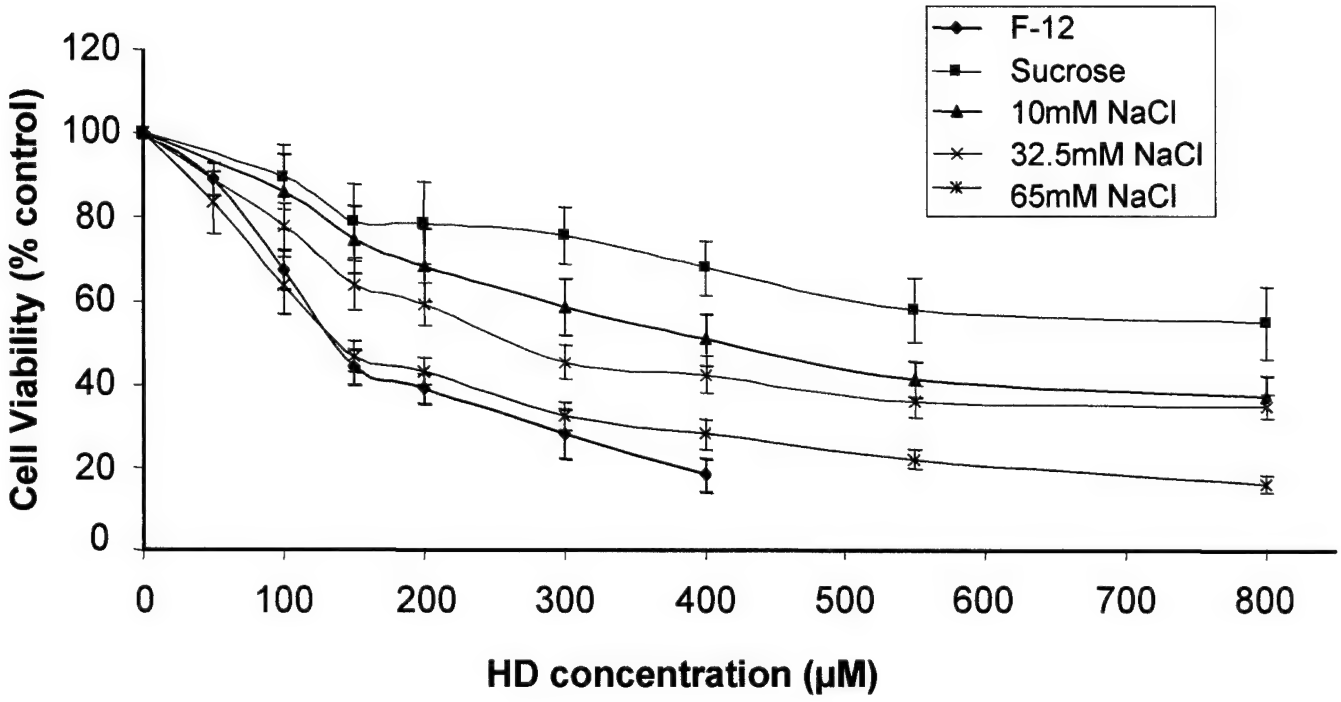

F-12
Sucrose
10mM NaCl
32.5mM NaCl
65mM NaCl
Cell Viability (% control)
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
HD concentration (μM)

Fig. 28

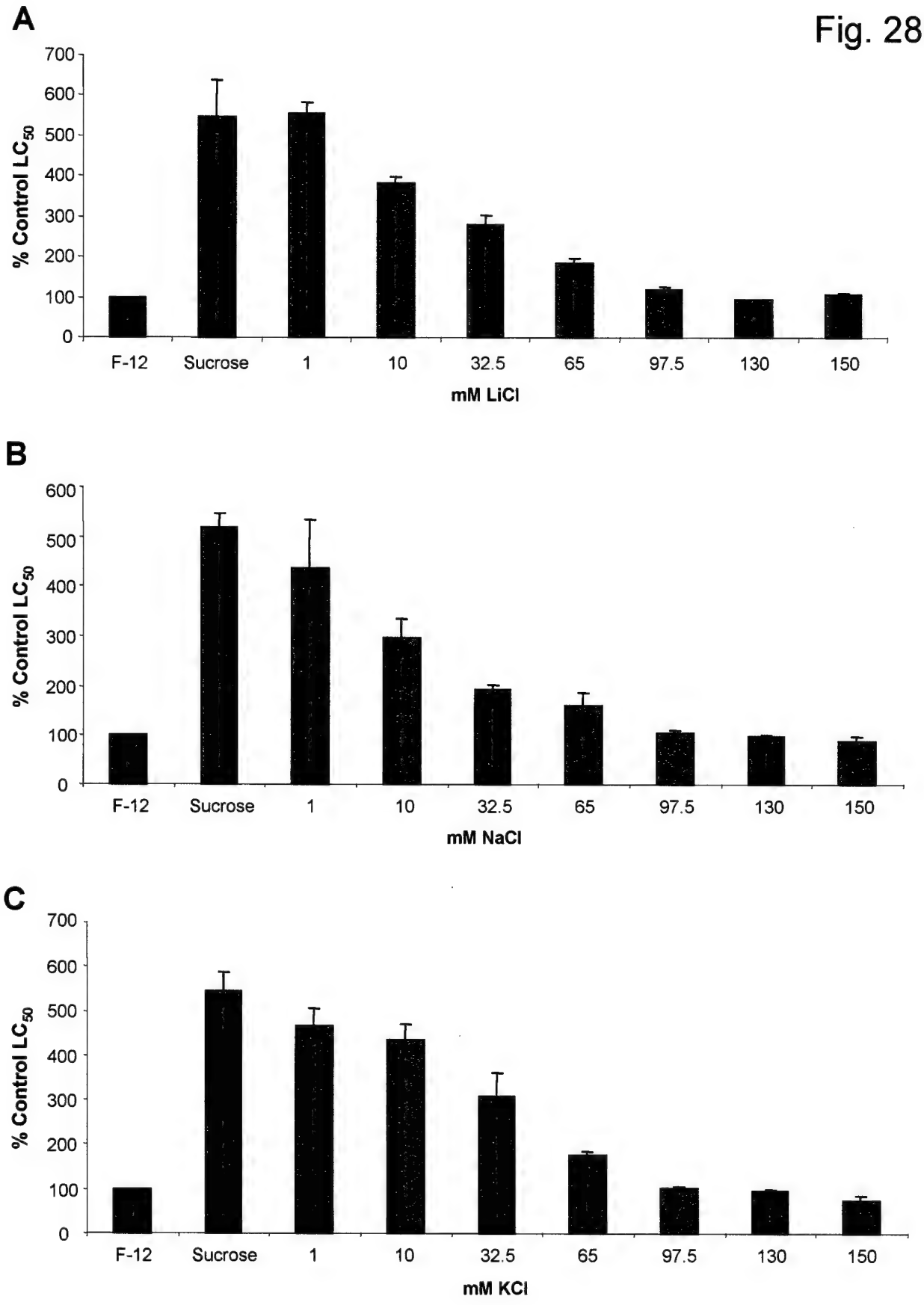

A
% Control $LC_{50}$
700
600
500
400
300
200
100
0
F-12
Sucrose
1
10
32.5
65
97.5
130
150
mM LiCl
B
% Control $LC_{50}$
600
500
400
300
200
100
0
F-12
Sucrose
1
10
32.5
65
97.5
130
150
mM NaCl
C
% Control $LC_{50}$
700
600
500
400
300
200
100
0
F-12
Sucrose
1
10
32.5
65
97.5
130
150
mM KCl

Fig. 29

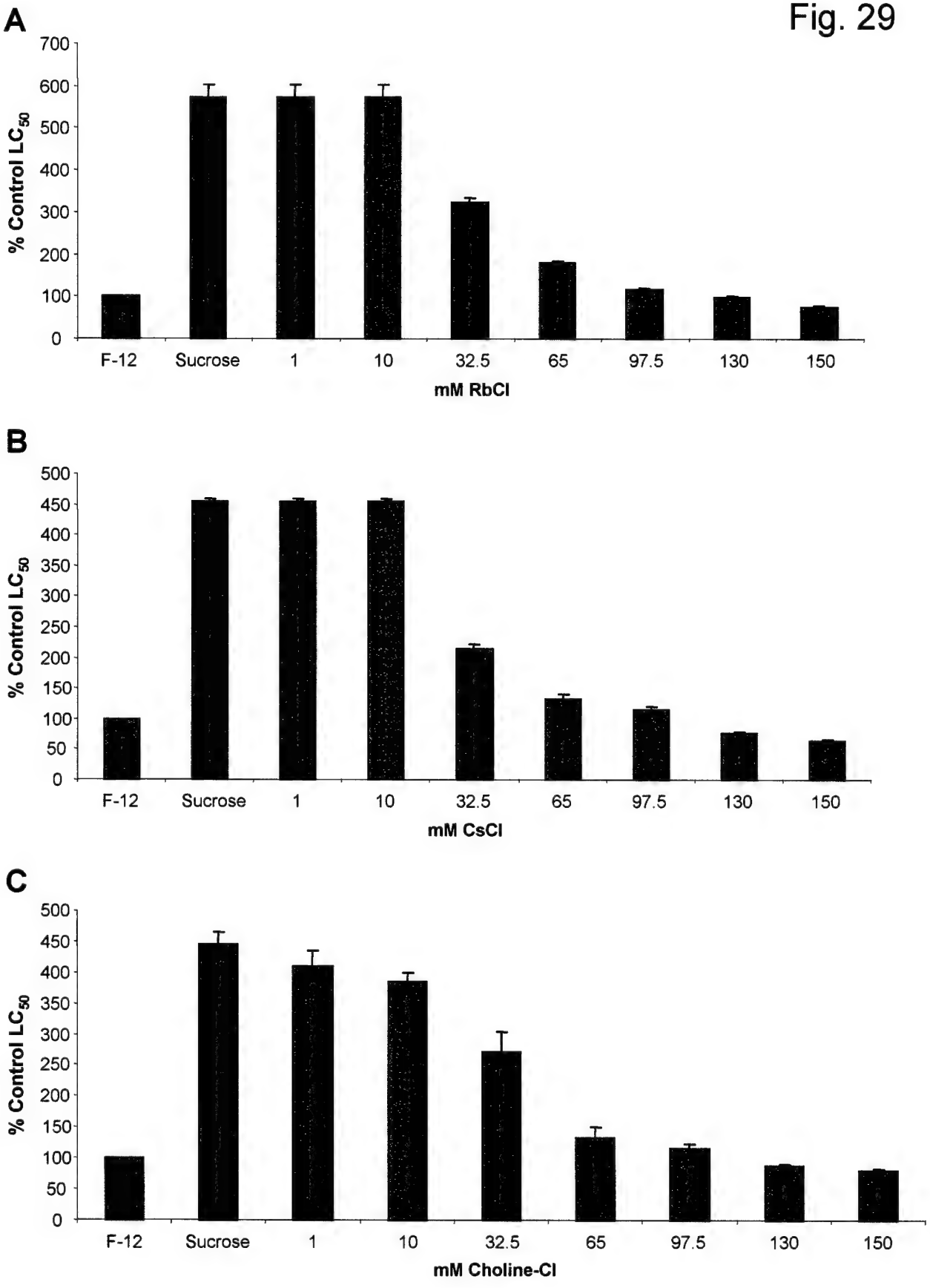

A
% Control LC50
700
600
500
400
300
200
100
0
F-12
Sucrose
1
10
32.5
65
97.5
130
150
mM RbCl
B
% Control LC50
500
450
400
350
300
250
200
150
100
50
0
F-12
Sucrose
1
10
32.5
65
97.5
130
150
mM CsCl
C
% Control LC50
500
450
400
350
300
250
200
150
100
50
0
F-12
Sucrose
1
10
32.5
65
97.5
130
150
mM Choline-Cl

Fig. 30

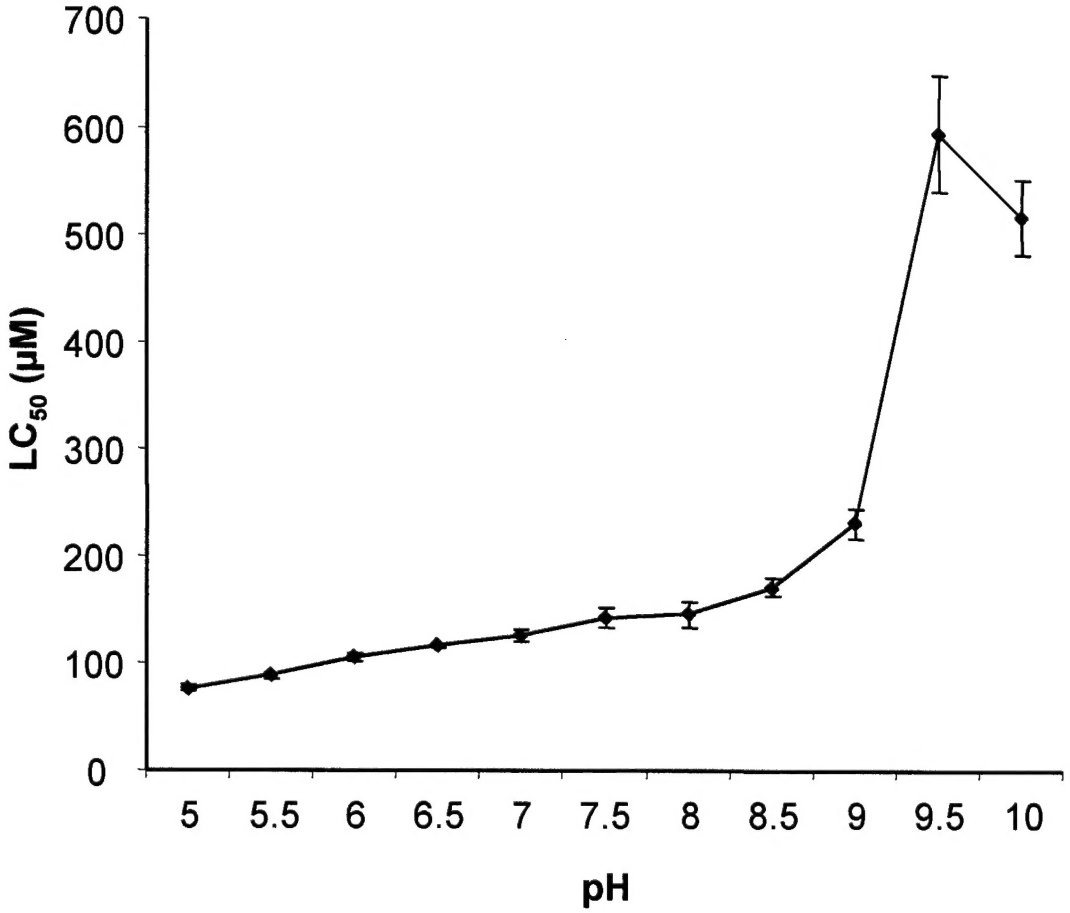


Fig. 31

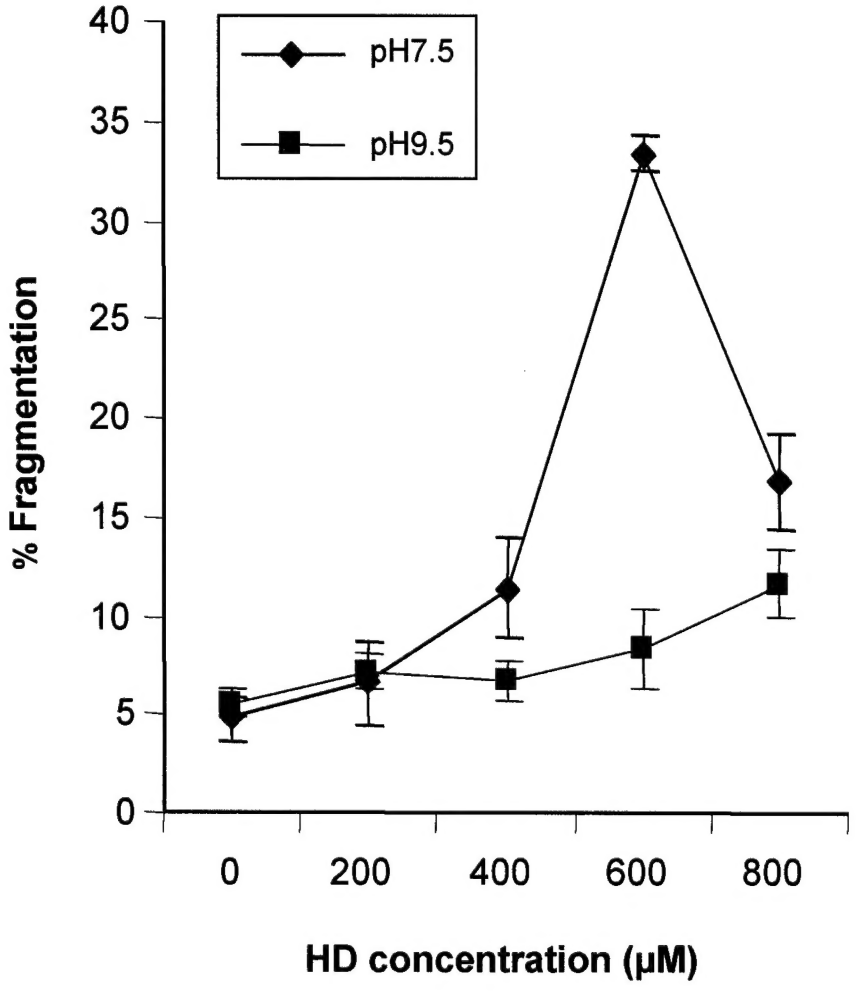

40
35
30
25
20
15
10
5
0
% Fragmentation
pH7.5
pH9.5
0
200
400
600
800
HD concentration (µM)

Fig. 32

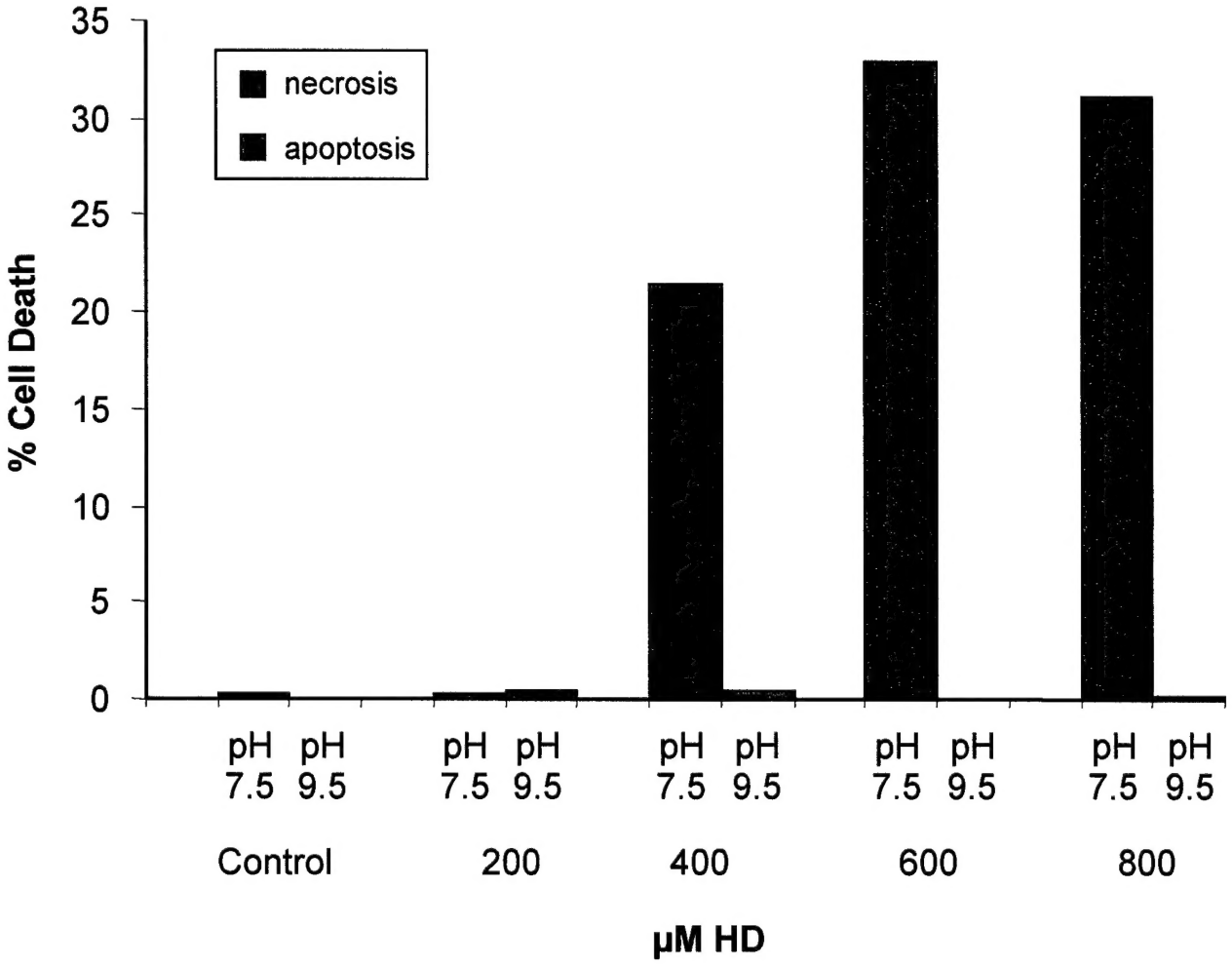

35
30
25
20
15
10
5
0
% Cell Death
necrosis
apoptosis
pH 7.5
pH 9.5
Control
200
400
600
800
µM HD

Fig. 33